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(54) Title: TRAIL: AN INHIBITOR OF AUTOIMMUNE INFLAMMATION AND CELL CYCLE PROGRESSION

(57) Abstract: The present invention provides a method for achieving normal levels of cellular apoptosis in non-transformed cells of a patient by administering to the patient a therapeutically effective amount of purified TRAIL ligand or active fragment thereof. In particular, such method is provided to a patient suffering from an autoimmune disease or condition, such as arthritis, encephalomyelitis or multiple sclerosis or autoimmune inflammation in the CNS. The present invention further provides a method of blocking the activity of an endogenous TRAIL receptor or inhibitor in a patient by administering to the patient a therapeutically effective amount of a purified TRAIL agonist, in an amount sufficient to enhance the patient's level of TRAIL ligand. In particular, such method is provided to enhance ameliorate or restore cellular apoptosis in non-transformed cells of the patient.

TRAIL: An Inhibitor of Autoimmune Inflammation and Cell Cycle Progression

REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application 60/157,222, filed September 30, 1999.

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FIELD OF THE INVENTION

The present invention is related to the genetic regulation of cellular apoptosis in nontransformed tissues, specifically the effect of Tumor necrosis factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL).

BACKGROUND OF THE INVENTION

In view of the potent effects of cellular apoptosis on growth, development, and homeostasis, it is not surprising to find that the initiation of apoptosis is tightly regulated, especially in normal cells.

TRAIL, the Tumor necrosis factor (TNF)-Related Apoptosis-Inducing Ligand, is a type II membrane protein of the TNF superfamily (Wiley *et al*, *Immunity* 3(6):673-682 (1995)). Some members of the TNF family are capable of inducing apoptosis of normal and/or tumor cells. Both TNF and CD95L have been shown to mediate activation-induced cell death (AICD) of lymphocytes (Ju *et al.*, *Nature* 373:444-448 (1995); Dhein *et al.*, *Nature* 373:438-441 (1995); Brunner *et al.*, *Nature* 373:441-444 (1995)). However, unlike other members of the TNF superfamily that interact with one or two specific receptors, TRAIL can potentially interact with five different receptors. These include Death Receptor 4 (DR4, TRAIL-R1), Death Receptor 5 (DR5, TRAIL-R2), Decoy Receptor 1 (DcR1, TRAIL-R3, TRID), Decoy Receptor 2 (DcR2, TRAIL-R4, TRUNDD) (Pan *et al.*, *Science* 277(5327):815-818 (1997); Pan *et al.*, *FEBS Lett.* 424(1-2):41-45 (1998); Schneider *et al.*, *FEBS Lett.* 416(3):329-334 (1997); Sheikh *et al.*,

Cancer Research 58(8):1593-1598 (1998); Sheridan *et al.*, *Science* 277(5327):818-821 (1997); Walczak *et al.*, *EMBO J.* 16(17):5386-5397 (1997); Screaton *et al.*, *Curr. Biol.* 7(9):693-696 (1997)), and a soluble receptor called osteoprotegerin (Emery *et al.*, *J.Biol.Chem.* 273(23): 14363-14367 (1998)).

While the presence of multiple TRAIL receptors strongly suggests that TRAIL could be involved in multiple processes, the precise roles of TRAIL in health and disease have, to date, been unknown. *In vitro* studies have shown that TRAIL induces apoptosis of some, but not all, tumor cell lines (Pan *et al.*, 1997; Sheridan *et al.*, 1997). This appears to be mediated by the death receptors, DR4 and DR5, which are capable of activating the caspase cascade.

The presence of DcR1 and DcR2 which possess similar intracellular death domains as TNF receptors and CD95 (Fas/Apo-1), and are capable of activating the caspase cascade. The presence of decoy receptors, DcR1 and DcR2, which do not contain functional death domains, blocks TRAIL-induced apoptosis (Pan *et al.*, 1997; Sheridan *et al.*, 1997).

Although both TRAIL and TRAIL receptors are constitutively expressed in various tissues and in a variety of cell types, including lymphocytes, natural killer cells, and neural cells (Wiley *et al.*, 1995; Pan *et al.*, 1997; Schneider *et al*, 1997; Rieger *et al.*, *FEBS Lett.* 427:124 (1998); Kayagaki *et al.*, *J. Immunol.* 163:1906 1999); Bretz *et al.*, J. D., *J. Biol. Chem.* 274:23627 (1999); Frank *et al., Biochem. Biophys. Research Comm.* 257:454 (1999); Sedger *et al.*, *J. Immunol.* 163:920 (1999); Wu *et al.*, *Cancer Research* 59(12):2770-2775 (1999)), and are upregulated upon cell activation (Sheikh *et al.*, 1998; Mariani *et al.*, *European J. Immunol.* 28(5):1492-1498 (1998); Jeremias *et al.*, *European J. Immunol.* 28(1):143-152 (1998)), TRAIL may not induce apoptosis of most non-transformed cells (Pan *et al.*, 1997; Sheridan *et al.*, 1997). *In vivo* administration of recombinant TRAIL selectively kills tumor cells, but not normal cells, leaving the host organ systems unharmed (Walczak *et al.*, *Nature Med.* 5(2):157-163 (1999); Ashkenazi *et al.*, *J. Clin. Invest.* 104(2):155-162 (1999)).

While this finding has generated tremendous interest in using recombinant TRAIL for cancer therapy, it has also raised fundamental questions regarding the roles of TRAIL in normal non-transformed tissues. Thus, there has been a need in the art to understand

TRAIL as a member of the TNF family, and as a potent inhibitor of autoimmune inflammation and cell cycle progression.

SUMMARY OF THE INVENTION

TRAIL, the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand, induces apoptosis of tumor cells, but not normal cells; yet its role in normal non-transformed tissues was, until the present invention, unknown. The present invention demonstrates that chronic blockade of TRAIL in mice exacerbates autoimmune rheumatoid arthritis in a representative animal model, while intra-articular TRAIL gene transfer ameliorates the disease. *In vivo*, TRAIL-blockade led to profound hyper-proliferation of synovial cells and arthritogenic lymphocytes, and heightened the production of cytokines and autoantibodies. *In vitro*, TRAIL inhibited DNA synthesis and prevented cell cycle progression of lymphocytes.

However, TRAIL had no effect on apoptosis of inflammatory cells either *in vivo* or *in vitro*. Thus, unlike Fas ligand or other members of the tumor necrosis factor superfamily, TRAIL is a prototype inhibitor protein that inhibits autoimmune inflammation by blocking cell cycle progression of T cells and inhibited their differentiation into effector cells, but it does not mediate activation–induced cell death of T lymphocytes.

The present invention also, for the first time, demonstrates the consequences of TRAIL-blockade in an animal model of multiple sclerosis. Indeed, confirming the effect in autoimmune diseases, as shown in arthritis, chronic TRAIL-blockade in mice exacerbated experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG). The exacerbation was evidenced primarily by increases in disease score and degree of inflammation in the central nervous system (CNS). Interestingly, the degree of apoptosis of inflammatory cells in the CNS was not affected by TRAIL-blockade, suggesting that TRAIL may not regulate apoptosis of inflammatory cells in EAE.

By contrast, MOG-specific TH1 and TH2 cell responses were significantly enhanced in animals treated with the soluble TRAIL receptor. Thus, unlike TNF, which promotes autoimmune inflammation, TRAIL inhibits autoimmune encephalomyelitis and

prevents activation of autoreactive T cells. Accordingly, unlike many other members of the TNF family that promote autoimmune encephalomyelitis, TRAIL inhibits EAE and prevents activation of encephalitogenic T cells.

The present invention provides a method for achieving normal levels of cellular apoptosis in non-tranformed cells of a patient by administering to the patient a therapeutically effective amount of purified TRAIL ligand or active fragment thereof. In particular, the present invention provides such method to a patient suffering from an autoimmune disease or condition.

The method is also provided in which normal levels of cellular apoptosis is achieved in non-tranformed cells of a patient by administering to the patient a therapeutically effective amount of an isolated nucleic acid sequence encoding TRAIL or active fragment thereof.

The present invention further provides a method of blocking the activity of an endogenous TRAIL receptor or inhibitor in a patient by administering to the patient a therapeutically effective amount of a purified TRAIL agonist, in an amount sufficient to enhance the patient's level of TRAIL ligand. In particular, the present invention provides such method to enhance ameliorate or restore cellular apoptosis in non-tranformed cells of the patient, by administering to the patient a therapeutically effective amount of an isolated nucleic acid sequence encoding said TRAIL agonist, in an amount sufficient to enhance the patient's level of TRAIL ligand.

The method is also provided in which normal levels of cellular apoptosis is achieved in non-tranformed cells of a patient by administering to the patient a therapeutically effective amount of an isolated nucleic acid sequence encoding a TRAIL agonist. Such TRAIL agonist is a receptor or inhibitor of a TRAIL receptor or inhibitor, wherein the agonist is selected from the group consisting of antibodies to TRAIL receptors or inhibitors, antisense molecules complimentary to TRAIL receptors or inhibitors, and any molecule which binds to or blocks TRAIL receptors or inhibitors.

It is an object of the foregoing methods to prevent, inhibit or decrease inflammation, tissue damage or injury related to an autoimmune disease or condition in a patient. Such autoimmune diseases or conditions include arthritis, autoimmune encephalomyelitis, insulin-dependent diabetes mellitus, hemolytic anemias, rheumatic

fever, thyroiditis, Crohn's disease, myasthenia gravis, glomerulonephritis, autoimmune hepatitis, multiple sclerosis, systemic lupus erythematosus, ankylosing spondylitis, and others. The methods are also useful for the treatment of autoimmune disease or conditions resulting from allogeneic tissue or organ transplant or graft-versus-host disease. In particular, the present methods are useful for the treatment or prevention of symptoms associated with arthritis, including induced, rheumatoid or chronic arthritis, or autoimmune encephalomyelitis or multiple sclerosis. The affected non-transformed cells include, but are not limited to, lymphocytes, including autoreactive lymphocytes, cytokines and synovial cells.

Additional objects, advantages and novel features of the invention will be set forth in part in the description, examples and figures which follow, and in part will become apparent to those skilled in the art on examination of the following, or may be learned by practice of the invention.

DESCRIPTION OF THE DRAWINGS

The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings, certain embodiment(s) which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities shown.

Figures 1A-1D are histograms depicting the blocking effect of recombinant sDR5 on TRAIL-induced apoptosis of Jurkat T leukemia tumor cells and K562 B cells. The Jurkat T leukemia cells were treated with the following: 5 μg/ml of BSA (Figure 1A), 100 ng/ml of TRAIL (Figure 1B), 5 μg/ml of sDR5 (Figure 1C), or 100 ng/ml of TRAIL plus 5 μg/ml of sDR5 (Figure 1D). Each histogram represents 10,000 events, with the apoptotic cells gated. Figure 1E graphically depicts dose-dependent killing of B lymphoma cells by TRAIL, with (open circle) or without (filled square) 5 μg/ml of sDR5.

Figures 2A and 2B graphically depict the effect of TRAIL-blockade *in vivo* in a mouse model of rheumatoid arthritis. Figure 2A depicts the disease course in mice treated with BSA (open square) or sDR5 (filled circle). Each data point represents a mean ± standard deviation (SD) from a total of 5 mice (for sDR5-treated group) or 6 mice

(for BSA-treated group). Figure 2B illustrates the dose-dependent effect of sDR5 on arthritis as judged by disease scores of individual feet taken 12 days after the second immunization. Four groups of mice are shown: one was treated with 100 μg BSA, while the other three were treated with 50-300 μg sDR5. Each data point represents an individual foot, with 16 to 24 feet per group. $P<0.05$ for mice treated with 50 μg sDR5 and $p<0.01$ for mice treated with 100 or 300 μg sDR5.

Figure 3 graphically depicts the ability various concentrations of recombinant TRAIL virus on arthritis following intra-articular injection, and the ability of sDR5 to neutralize the arthritis-ameliorating effect as measured by the return of the disease.

Figures 4A-4H depict the histochemical profiles of arthritic joints of mice treated as in Figure 2. Figure 4A depicts ankle joint of BSA-treated mouse with a pathology score of 2 (HE staining, original magnification x 20). Arrow indicates signs of synovitis. Figure 4B depicts ankle joint of sDR5-treated mouse with a pathology score of 4 (HE staining, original magnification x20). Arrows indicate severe synovitis, hyperplasia, cartilage and bone destruction. Figure 4C depicts ankle joint of BSA-treated mouse with a pathology score of 1 (HE staining, original magnification x100). Arrow indicates signs of synovitis. Figure 4D depicts ankle joint of sDR5-treated mouse with a pathology score of 4 (HE staining, original magnification x100). Arrows indicate severe synovitis, hyperplasia, cartilage and bone destruction. Figure 4E depicts ankle joint of BSA-treated mouse with a disease score of 2 (BrdU staining, original magnification x400). Arrows indicate $BrdU^+$ nuclei. Figure 4F depicts ankle joint of sDR5-treated mouse with a disease score of 3 (BrdU staining, original magnification x 400). Figure 4G depicts ankle joint of BSA-treated mouse with a pathology score of 4 (apoptotic staining, original magnification x200). Arrows indicate apoptotic cells. Figure 4H depicts ankle joint of sDR5-treated mouse with a pathology score of 4 (apoptotic staining, original magnification x 200).

Figures 5A and 5B graphically depict the quantitative analysis corresponding to the histochemical data displayed in Figure 4, comparing an sDR5-treated group of mice with a BSA-treated group. Figure 4A presents the pathology scores revealed significant differences between the two groups, as determined by ANOVA ($p<0.01$). Figure 4B

presents the apoptotic index, wherein the differences between the two groups are not statistically significant, as determined by ANOVA ($p = 0.21$).

Figures 6A-6E graphically depict the effect of TRAIL-blockade on anti-collagen immune responses in mice treated as in Figure 2A. Figure 6A depicts lymphocyte proliferative responses as determined by ^{3}H-thymidine incorporation. Figure 6B depicts IL-2 production; Figure 6C depicts IFN-γ production. Each data point represents a mean ± SD from 5 (for sDR5-treated group) or 6 (for BSA group) mice. Radioactivity is presented as counts per minute (CPM). To test humoral immune responses, anti-collagen IgG2a antibodies (Figure 6D) and IgG1 antibodies (Figure 6E) were determined by ELISA using chicken type II collagen as antigen. The experiments were repeated three times with similar results. Open bar = mice treated with BSA; filled bar = mice treated with sDR5.

Figures 7A-7D graphically depict inhibition of DNA synthesis and cell cycle progression by TRAIL on splenocytes prepared from BALB/c mice. Purified live cells were cultured with or without the following reagents: 1) 100 ng/ml of TRAIL; 2) 5 μg/ml of sDR5; 3) 5 μg/ml of anti-CD95L mAb (MFL-3), and 4) anti-mouse CD3 mAb. Figure 7A shows the percentage of apoptotic cells as determined by flow cytometry. The differences between anti-CD95L mAb treated culture and all other cultures are statistically significant as determined by ANOVA ($p<0.0001$). Figure 7B depicts the number of S-G2/M cells/well, as determined by flow cytometry. The total numbers of live cells/well recovered from each group were as follows: cultures with anti-CD3 mAb alone, 0.3×10^5; cultures with anti-CD3 mAb + sDR5, 1.6×10^5; cultures with anti-CD3 mAb + anti-CD95L mAb, 1.1×10^5; cultures with anti-CD3 mAb + sDR5 + TRAIL, 0.6×10^5. The differences between all four groups are statistically significant as determined by ANOVA ($p<0.01$). Figures 7C and 7D depict DNA synthesis as determined by ^{3}H-thymidine incorporation. For cultures containing anti-CD3 mAb, the differences between the two groups are statistically significant as determined by ANOVA ($p<0.01$).

Figure 8 graphically depicts the blocked TRAIL-induced apoptosis of mouse L929 cells by recombinant sDR5. L929 cells were treated with different concentrations of recombinant TRAIL with or without sDR5.

Figures 9A and 9B graphically depict exacerbation of EAE by TRAIL-blockade. In Figure 9A, the sDR5 injections were performed 7 days after disease onset until the end of the experiment; whereas in Figure 9B, the sDR5 injections were performed from the day of immunization to the day of disease onset (17 days after immunization). Only mice that developed EAE were included for calculating mean clinical scores. The differences between the two groups are statistically significant as determined by Mann-Whitney test ($P<0.01$) for panel A, but not for B.

Figures 10A-10D depict inflammation and apoptosis in the spinal cord. Mice were treated as in Figure 9A and sacrificed 42 days after immunization. Spinal cord was treated and examined for histology and apoptosis. Figures 10A and 10B depict Luxol fast blue staining of spinal cords from sDR5 (Figure 10A) and BSA (Figure 10B) treated mice (original magnification x 100). Figures 10C and 10D depict TUNEL staining of spinal cords from sDR5 (Figure 10C) and BSA (Figure 10D) treated mice (original magnification x 200). Arrows indicates apoptotic nuclei.

Figure 11 graphically depicts quantitative analysis of the degree of inflammation in the CNS. Spinal cord was treated and examined for histology. Each data point represents a percentage of spinal cord section that is inflamed. The horizontal bars represent the means of respective groups. The differences between the two groups are statistically significant as determined by Student *t* test ($p<0.05$). Filled squares = mice treated with HSA. Open circles = mice treated with sDR5.

Figure 12 graphically depicts quantitative analysis of the degree of apoptosis in the CNS. Spinal cord was treated and examined for apoptosis. The numbers of apoptotic nuclei per mm^2 of inflamed tissue were counted and plotted against the percentages of the corresponding spinal cord sections that were inflamed. Filled squares = mice treated with HSA. Open circles = mice treated with sDR5.

Figure 13 depicts MOG-specific proliferation and cytokine production *in vitro*.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

The TNF family of proteins play crucial roles in a number of biological processes including apoptosis, immunity, inflammation and development. TRAIL is a newly identified member of the TNF family. Although it has been established that TRAIL,

unlike TNF, induces apoptosis of tumor cells, but not normal cells, the roles of TRAIL in health and disease have, to date, been virtually unknown. However, both TRAIL and TRAIL receptors are expressed in normal non-transformed tissues.

As is known to those of skill in the art, apoptosis is an active process of gene-directed cellular self-destruction. The present invention provides compositions and methods for controlling or enhancing normal levels of apoptosis in a suitable cell or a population of suitable cells by introducing into the cell or cells an effective amount of a nucleic acid molecule coding for a gene product having TRAIL biological activity, or for inhibiting or reducing TRAIL receptor or inhibitor activity. The method also may be practiced using the gene product itself. Accordingly, this method provides an improvement over prior art methods, wherein apoptosis can be controlled or enhanced by affecting the induction pathway at the level of ligand induction, such as by blocking or inhibiting TRAIL receptors or inhibitors, including antibodies or anti-ligand antibodies that interfere with the binding of the ligand to its cell surface receptor. Moreover, this invention can be combined with the use of prior art methods, known to affect apoptosis.

The present invention shows that TRAIL is a potent inhibitor of autoimmune conditions, such as arthritis or multiple sclerosis or autoimmune inflammation in the CNS. Blocking endogenous TRAIL with sDR5 eliminates this inhibition, and exacerbates autoimmune arthritis or encephalomyelitis. Furthermore, TRAIL apparently inhibits activation of autoreactive T cells that initiate autoimmune inflammation. The inhibitory effect of TRAIL on arthritis appears to result from inhibition of cell cycle progression and/or cytokine production. Blocking endogenous TRAIL with sDR5 eliminates this inhibition, and enhances proliferation of autoreactive lymphocytes (as shown in Figure 6A) or synovial cells (as shown in Figure 4). This may in turn contribute to the exacerbation of arthritic inflammation and joint tissue destruction.

Thus, one of the functions of TRAIL *in vivo* is to maintain immune homeostasis and to down-regulate immune responses including autoimmune responses. This discovery of the present invention is a startling contrast to TNF, which initiates and exacerbates autoimmune diseases. In fact, anti-TNF therapy is effective in preventing arthritic inflammation both in humans and animals (Maini *et al.*, *Immunol. Rev.* 144:195-223 (1995); Joosten *et al.*, *Arthritis & Rheumatism* 39(5):797-809 (1996); Williams *et al.*,

Proc. Natl. Acad. Sci. USA 89(20):9784-9788 (1992); Eliaz *et al.*, *Cytokine* 8(6):482-487 (1996)). Other members of the TNF family that have been reported to inhibit autoimmune inflammation are CD95 ligand (CD95L, Fas) and CD30 ligand (CD30L).

The findings reported in the present invention indicate that the role of TRAIL in autoimmunity may be more closely related to that of CD95L or CD30 ligand (CD30L). Mutations in CD95/CD95L genes lead to the development of systemic autoimmune diseases in both humans (Bettinardi *et al.*, *Blood* 89(3):902-909 (1997); Sneller *et al.*, *Blood* 89(4):1341-1348 (1997)) and mice (Nagata *et al.*, *Science* 267:1449-1456 (1995); Cohen *et al.*, *Immunol. Today* 13(11):427-428 (1992); Nagata *et al.*, *Immunol. Today* 16(1):39-43 (1995); Mountz *et al.*, *Int'l. Rev. Immunol.* 11(4):321-342 (1994)), although paradoxically they also prevent several organ-specific autoimmune diseases (Waldner *et al.*, *J. Immunol.*159:3100-3103 (1997); Sabelko *et al.*, *J. Immunol.* 159:3096-3099 (1997); Giordano *et al.*, *Science* 275(5302):960-963 (1997); Chervonsky *et al.*, *Cell* 89(1):17-24 (1997); Kang *et al.*, *Nature Medicine* 3(7):738-743 (1997); Muruve *et al.*, *Human Gene Therapy* 8(8):955-963 (1997); Itoh *et al.*, *J. Exp. Med.* 186(4):613-618 (1997)).

Up-regulating CD95 or CD95L function in synovial joints has been reported to ameliorate autoimmune arthritis (Zhang *et al*, 1997; Fujisawa *et al.*, *J. Clin. Invest.* 98(2):271-278 (1996)). Similarly, CD30L may also play an anti-inflammatory role in autoimmune diseases (Kurts *et al.*, *Nature* 398(6725):341-344 (1999)). Autoreactive $CD8^+$ T cells deficient in CD30L elicits more severe autoimmune insulitis in mice (Kurts *et al.*, 1999). Thus, unlike TNF, but similar to CD95L and CD30L, TRAIL appears to be a member of an inhibitor protein subfamily that prevents autoimmune diseases by downregulating immune responses.

However, the present findings indicate that the mechanism of TRAIL action *in vivo* is different from that of CD95L. While CD95L induces apoptosis of activated T cells, TRAIL appears to inhibit their proliferation without eliminating them through apoptosis. This finding is consistent with recent reports that, unlike CD95L, TRAIL induces apoptosis of tumor cells but not normal cells (Pan *et al.*, 1997; Sheridan *et al.*, 1997). Systemic administration of recombinant TRAIL, but not CD95L, selectively kills tumor cells while sparing normal host cells Walczak *et al.*, 1999; Ashkenazi *et al.*, 1999).

Thus, the observation in the present invention that TRAIL can inhibit DNA synthesis provides direct evidence that TRAIL can prevent G1-to-S phase progression of lymphocytes. Therefore, unlike TNF or CD95L, TRAIL inhibits activation and expansion of lymphocytes *in vivo*, but does not delete them from the system.

The present invention further demonstrates that autoreactive T cell activation was enhanced in mice treated with sDR5, suggesting that TRAIL may inhibit functions of autoreactive T cells. Since EAE is a T cell mediated autoimmune disease, inhibiting T cell function may diminish the disease. However, it should also be pointed out that enhancing T cell function alone during the inductive phase of EAE may not be sufficient to exacerbate the disease, since treating mice with sDR5 before the onset of the disease failed to significantly affect EAE (Figure 9B). A similar effect of TRAIL was seen in another model of autoimmunity, *i.e.*, collagen-induced arthritis in DBA/1 mice (Song *et al.*, 2000). As shown in the examples that follow, TRAIL-blockade during the effector phase of the disease enhanced, for example, arthritic inflammation.

In an alternative mechanism, TRAIL may inhibit autoimmune inflammation by inducing apoptosis of inflammatory cells. Although it has been shown that TRAIL does not induce apoptosis of most non-transformed cell, there is evidence to suggest that dendritic cells and some T cells may be susceptible to TRAIL-induced apoptosis *in vitro* (Jeremias *et al.*, *European J. Immunol.* 28:143 (1998); Wang *et al.*, *Cell* 98:47 (1999)). The demonstration in the present invention that the degrees of apoptosis in the CNS and arthritic joints were not affected by TRAIL-blockade suggests that TRAIL may not regulate apoptosis of inflammatory cells in these systems.

Thus, by *in vivo* TRAIL-blockade, it is clearly established in the present invention that, unlike TNF, TRAIL inhibits autoimmune encephalomyelitis and prevents activation of autoreactive T cells. Since EAE is an animal model for human multiple sclerosis and since TRAIL and its receptors are also expressed by human cells, the present results are important, not only for understanding the pathogenesis of EAE, but also for designing therapeutic strategies for the treatment of autoimmune diseases such as multiple sclerosis.

It has been reported that some of the TRAIL receptors can activate both caspase (through FADD/TRADD) and NF-kappaB pathways in tumor cells (Schneider *et al.*, *Immunity* 7(6):831-836 (1997); Degli-Esposti *et al.*, *Immunity* 7(6):813-820 (1997);

Chaudhary *et al.*, *Immunity* 7(6):821-830 (1997)). It is, therefore, necessary to determine whether this also occurs in normal cells, and if so, whether this is responsible for the cell cycle arresting effect reported in the present invention. It is to be noted that NF-kappaB activation occurs in collagen-induced arthritis, and that inhibition of NF-kappaB activation in T cells prevents the disease (Seetharaman *et al.*, *J. Immunol.* 163(3):1577-1583 (1999)). On the other hand, various signal transduction pathways of TRAIL receptors are yet to be defined. Novel unidentified pathways may be responsible for the inhibitory effect of TRAIL in inflammation and cell cycle progression.

In sum, the regulation of programmed cell death is vital for normal functioning of the immune system. For example, T cells that recognize self-antigens are destroyed through the apoptotic process during maturation of T-cells in the thymus, whereas other T cells are positively selected. Insufficient apoptosis has been implicated in certain conditions, while elevated levels of apoptotic cell death have been associated with other diseases. The desirability of identifying and using agents that regulate apoptosis in treating such disorders is recognized (Kromer, *Adv. Immunol.*, 58:211 (1995).

Abnormal resistance of T cells toward apoptosis has been linked to lymphocytosis, lymphadenopathy, splenomegaly, accumulation of self-reactive T cells, autoimmune disease, development of leukemia, and development of lymphoma (Kromer, 1995). Conversely, excessive apoptosis of T cells has been suggested to play a role in lymphopenia, systemic immunodeficiency, and specific immunodeficiency, with specific examples including

virus-induced immunodeficient states associated with infectious mononucleosis and cytomegalovirus infection, and tumor-mediated immunosuppression (Kromer, 1995).

Since TRAIL binds and kills leukemia cells (the Jurkat cell line), TRAIL also may be useful in treating leukemia. A therapeutic method involves contacting leukemia cells with an effective amount of TRAIL. In one embodiment, a leukemia patient's blood is contacted *ex vivo* with an TRAIL polypeptide. The TRAIL may be immobilized on a suitable matrix. TRAIL binds the leukemia cells, thus removing them from the patient's blood before the blood is returned into the patient. Acted with an amount of TRAIL effective in inducing death of leukemia cells in the bone marrow. Following use of TRAIL to purge leukemia cells, the thus-treated marrow is returned to the patient.

TRAIL also binds to, and induces apoptosis of, lymphoma and melanoma cells (US Patent No. 5,763223, hence therapeutic approaches using TRAIL may be applicable to lymphoma and melanoma cells. TRAIL polypeptides may be employed in treating cancer, including, but not limited to, leukemia, lymphoma, and melanoma.

In the alternative, rather than utilizing an effective amount of TRAIL, endogenous levels of TRAIL may be restored in the patient, presumably therapeutically effective levels, by binding or inhibiting TRAIL receptors or inhibitors.

TRAIL polypeptides also find use in treating viral infections. Contact with TRAIL has been shown to cause death of cells infected with cytomegalovirus, but not of the same cell type when uninfected. Such viruses include, but are not limited to, encephalomyocarditis virus, Newcastle disease virus, vesicular stomatitis virus, herpes simplex virus, adenovirus-2, bovine viral diarrhea virus, HIV, and Epstein-Barr virus.

An effective amount of TRAIL is administered to a mammal, including a human, afflicted with a viral infection. In one embodiment, TRAIL is employed in conjunction with interferon to treat a viral infection, such as γ-interferon pretreatment of CMV-infected cells to enhance the level of killing of the infected cells that was mediated by TRAIL. TRAIL may be administered in conjunction with other agents that exert a cytotoxic effect on cancer cells or virus-infected cells.

In another embodiment, TRAIL is used to kill virally infected cells in cell preparations, tissues, or organs that are to be transplanted. For example, bone marrow may be contacted with TRAIL to kill virus-infected cells that may be present therein, before the bone marrow is transplanted into the recipient.

The TRAIL of the present invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective or insufficient amounts of TRAIL. A therapeutically effective amount of purified TRAIL protein is administered to a patient afflicted with such a disorder. Alternatively, TRAIL DNA sequences may be employed in developing a gene therapy approach to treating such disorders. Disclosure herein of native TRAIL nucleotide sequences permits the detection of defective TRAIL genes, and the replacement thereof with normal TRAIL-encoding genes. Defective genes may be detected by *in vitro* diagnostic assays, and by comparision of the native TRAIL

nucleotide sequence disclosed herein with that of a TRAIL gene derived from a person suspected of harboring a defect in this gene.

TRAIL DNA and polypeptides encoded thereby may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, TRAIL. TRAIL polypeptides may be administered to a mammal afflicted with such a disorder. Alternatively, a gene therapy approach may be taken.

The TRAIL protein employed in the pharmaceutical compositions preferably is purified, meaning that the TRAIL protein is substantially free of other proteins of natural or endogenous origin, desirably containing less than about 20%, preferably less than 10%, more preferably less than 5%, most preferably less than 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics.

Another use of the findings of the present invention is as a research tool for studying the biological effects that result from inhibiting TRAIL/TRAIL blockade interactions on different cell types. TRAIL blockade *in vitro* or *in vivo* may be achieved by a variety of known procedures in the art. For example, a purified TRAIL receptor polypeptide, such as that taught by US Patent No. 6,072,047, may be used to inhibit binding of TRAIL to endogenous cell surface TRAIL receptors. Biological effects that result from the binding of TRAIL to endogenous receptors thus are inhibited. Various forms of TRAIL receptors may be employed, including, for example, the TRAIL receptor fragments, oligomers, derivatives, and variants that are capable of binding TRAIL, including soluble TRAIL receptor used to inhibit a biological activity of TRAIL, *e.g.*, to inhibit TRAIL-mediated apoptosis of particular cells.

Blocking TRAIL receptors or inhibitors or restoring normal levels of TRAIL to a mammal may mediate TRAIL-deficit disorders. Such TRAIL-deficit disorders include conditions caused (directly or indirectly) or exacerbated by blockage of, removal of, or significantly diminished levels of TRAIL expression in the patient. In the alternative, soluble TRAIL may be used to treat those patients suffering from an insufficiency of normal levels of the TRAIL polypeptide, necessary to maintain normal levels of apoptosis needed to minimize inflammation in autoimmune conditions.

TRAIL, including soluble TRAIL may be employed in conjunction with other agents useful in treating a particular disorder. Normal levels of TRAIL-mediated cell death may be restored by conventional procedures, including T cell death, believed to occur through the mechanism known as activation-induced cell death (AICD).

Autoimmune diseases and conditions in which TRAIL-mediated cell death plays a significant role include, but are not limited to, *e.g.*, rheumatoid arthritis, insulin-dependent diabetes mellitus, hemolytic anemias, rheumatic fever, thyroiditis, Crohn's disease, myasthenia gravis, glomerulonephritis, autoimmune hepatitis, multiple sclerosis, systemic lupus erythematosus, ankylosing spondylitis and others. The present invention is also applicable to patients who have received, or who are about to receive, an allogeneic tissue or organ transplant, such as an allogeneic kidney, liver, heart, skin, bone marrow, as well as those experiencing graft-versus-host disease. Treatment of a patient at an early stage of an autoimmune disease will minimize or eliminate deterioration of the disease state into a more serious condition.

In one embodiment, a patient's blood or plasma is contacted with TRAIL *ex vivo* to remove TRAIL receptors or inhibitors. The TRAIL may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL bound to the matrix, before being returned to the patient. The immobilized TRAIL binds TRAIL receptors or inhibitors, thus removing TRAIL receptor or inhibitors from the patient's blood to restore normal levels of controlled cell apoptosis, for example to reduce inflammation in autoimmune disease patients.

In treating patients to restore normal levels of controlled cell apoptosis, TRAIL may be employed in combination with other effectors of T cell apoptosis, or inhibitors of TRAIL receptors or inhibitor. Such inhibitors of TRAIL receptors or inhibitors could include antibodies to TRAIL receptors or inhibitors, or antisense molecules capable of binding same.

Compositions comprising an effective amount of a TRAIL polypeptide of the present invention, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. TRAIL can be formulated according to known methods used to prepare pharmaceutically useful compositions.

TRAIL can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (*e.g.*, saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (*e.g.*, thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in Remington's Pharmaceutical Sciences.

In addition, such compositions can contain TRAIL complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of TRAIL, and are thus chosen according to the intended application. TRAIL expressed on the surface of a cell may find use, as well.

Compositions of the present invention may contain a TRAIL polypeptide in any form described herein, such as native proteins, variants, derivatives, oligomers, and biologically active fragments. In particular embodiments, the composition comprises a soluble TRAIL polypeptide or an oligomer comprising soluble TRAIL polypeptides.

In the alternative, the gene or an active fragment of the gene encoding the TRAIL polypeptide can be introduced into the patient, thereby permitting TRAIL to be expressed by, or enhancing the expression of, TRAIL by the patient, at a level not possible prior to introduction of the gene or gene fragment. The gene may be introduced by any known method, including gene therapy. As used herein, the phrase "gene therapy" refers to the transfer of genetic material (*e.g.*, DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition. The genetic material of interest encodes a product (*e.g.*, the TRAIL protein, polypeptide, peptide or functional RNA) whose production *in vivo* is desired.

As used herein, the term "introduce" or "introducing" in relation to nucleic acid encompasses any method of inserting a heterologous or exogenous nucleic acid molecule, such as the molecule encoding TRAIL, into a cell and includes, but is not limited to

transduction, transfection, microinjection and viral infection of host cells. Methods of carrying out these procedures are well known to those of skill in the art.

A "gene" is defined as any nucleic acid sequence that encodes an active or functional molecule. As used herein, the term "nucleic acid" means DNA, including cDNA, or RNA. A "cDNA clone" refers to a clone containing a DNA insert that was synthesized from mRNA and does not contain introns. The gene may encode therapeutic molecules including TRAIL, antisense or ribozyme RNAs, a gene encoding an enzyme, a gene encoding a cytokine or other immune modulating macromolecule, a gene encoding a recombinant antibody, a gene encoding a vaccine antigen, a gene encoding a macromolecule which complements genetic defects in somatic cells, and the like.

A "recombinant" DNA molecule or a "hybrid" DNA refers to a molecule consisting of segments of DNA from different genomes, which have been joined end-to-end outside of living cells, and which can be maintained in living cells.

Isolated nucleic acids useful in this invention are those that contain substantially no additional nucleic acids, and encode a polypeptide functionally equivalent to a polypeptide encoded by the isolated TRAIL gene when expressed under the control of necessary expression control sequences, of the types well known in the art of recombinant genetic technology.

"Transcription" refers to the process of producing mRNA from a gene or DNA sequence. "Translation" refers to the process of producing a polypeptide from mRNA. "Expression" refers to the process undergone by a gene or DNA sequence to produce a polypeptide, meaning a combination of transcription and translation. "Expression control sequences" are those nucleotide sequences that control and regulate expression of genes when operatively linked to those genes, such as promoters, enhancers and the like.

Various expression control sequences may also be chosen to effect the expression of the DNA sequences of this invention. These expression control sequences include those listed above and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses and various combinations thereof. For expression of the DNA sequences of the present invention, these DNA sequences are operatively-linked to one or more of the above-described expression control sequences in the expression vector. Such operative linking, which may be effected before or after the

chosen polypeptide DNA sequence is inserted into a cloning vehicle, enables the expression control sequences to control and promote the expression of the DNA sequence.

As used herein the term "functionally equivalent nucleotide sequence" is intended to cover minor variations in the viral vector sequence which, due to degeneracy in the DNA code, does not result in a peptide having substantially different biological activities from the native TRAIL peptide. The encoded TRAIL proteins can have an amino acid sequence, which is at least in part different from the native virus sequences, but which should retain substantially the same biological activities as the native TRAIL. This may be achieved by various changes in the sequence, such as insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the peptide produce.

TRAIL or active fragments or derivatives thereof, or the gene or gene fragment encoding TRAIL, can be administered to a patient in a therapeutically effective amount by any suitable manner, *e.g.*, topically, parenterally, or by inhalation. The term "parenteral" includes injection, *e.g.*, by subcutaneous, intravenous, or intramuscular routes, also including localized administration, *e.g.*, at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices. Compositions comprising TRAIL nucleic acids in physiologically acceptable formulations are also contemplated. TRAIL DNA may be formulated for injection, for example.

By "therapeutically effective" as used herein, is meant that amount of composition or expression product that is of sufficient quantity to enhance or ameliorate cellular apoptosis or to restore it to normal levels, particularly in nontransformed cells and/or tissues *in vivo*, preferably by modulating the level of TRAIL available to the cell as described above. The effect may be seen as a change in immunity, inflammation, particularly autoimmune inflammation, or development. By "ameliorate" is meant a

lessening or reduction or prophylactic prevention of the detrimental effect of the disorder in the patient receiving the therapy.

The terms "preventing" or "inhibiting" are intended to mean a reduction in effect on TRAIL, such as inhibiting a TRAIL receptor, thereby permitting endogenous or introduced TRAIL to reach normal levels in the patient. They also are intended to mean a diminution in the effect in a non-transformed cell of the morphological and/or biochemical changes normally associated with necessary and desirable levels of apoptosis, particularly in autoimmune diseases and conditions. Thus, this invention provides compositions and methods to modulate survival time and/or survival rate of a cell or population of cells which, absent the use of the method, will die, but would not be removed by controlled apoptosis. Accordingly, it also provides compositions and methods to prevent or treat diseases or pathological conditions associated with uncontrolled cell death in a subject, or to block TRAIL receptors or inhibitors that would otherwise preclude normal or desirable expression of the TRAIL peptide, which has been found related to normal levels of apoptosis in non-transformed cells, and which controls excessive inflammation, tissue injury and the like.

The method is provided in which normal levels of cellular apoptosis is achieved in non-tranformed cells of a patient by administering to the patient a therapeutically effective amount of an isolated nucleic acid sequence encoding a TRAIL agonist. Such TRAIL "agonist" is a receptor or inhibitor of a TRAIL receptor or inhibitor, wherein the agonist is selected from the group consisting of antibodies to TRAIL receptors or inhibitors, antisense molecules complimentary to TRAIL receptors or inhibitors, and any molecule which binds to or blocks TRAIL receptors or inhibitors.

The subject of the invention is preferably a human, however, it can be envisioned that any bird, animal, fish or the like, with a autoimmune or apoptotic condition can be treated by the method of the present invention.

Antibodies that are immunoreactive with TRAIL receptor or inhibitor polypeptides are also provided. Such antibodies specifically bind TRAIL receptors or inhibitors, in that the antibodies bind to TRAIL receptors or inhibitors via the antigen-binding sites of the antibody (as opposed to non-specific binding).

TRAIL receptors or inhibitors may be employed as an immunogen in producing antibodies immunoreactive therewith. Alternatively, another form of TRAIL receptor or inhibitor, such as a fragment or fusion protein, is employed as the immunogen.

Polyclonal and monoclonal antibodies may be prepared by conventional techniques.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann *et al.* (*Nature* 332:323 (1988)), Liu *et al.*, (*Proc. Nat'l. Acad. Sci.* 84:3439 (1987)), Larrick *et al.* (*Bio/Technology* 7:934 (1989)), and Winter *et al.* (*TIPS* 14:139 (1993)).

Among the uses of the antibodies is use in assays to detect the presence of TRAIL, or of TRAIL receptor or inhibitor polypeptides, either *in vitro* or *in vivo*. The antibodies also may be employed in purifying TRAIL, or TRAIL receptor or inhibitor, proteins by immunoaffinity chromatography.

Those antibodies that additionally can block the binding of TRAIL may be used to inhibit a biological activity that results from such binding. Such blocking antibodies to the TRAIL receptor or inhibitor may be identified using any suitable assay procedure, such as by testing antibodies for the ability to inhibit binding of TRAIL receptors or inhibitors. Examples of such cells are the Jurkat cells and PSI cells, and others.

Alternatively, blocking antibodies may be identified in assays for the ability to inhibit a biological effect that results from binding of TRAIL receptors or inhibitors to target cells. Antibodies may be assayed for the ability to inhibit TRAIL-mediated lysis of Jurkat cells, for example.

Such an antibody may be employed in an *in vitro* procedure, or administered *in vivo* to inhibit a TRAIL receptor or inhibitor-mediated biological activity. Disorders caused or exacerbated (directly or indirectly) by the interaction of TRAIL receptors or inhibitors with cell surface TRAIL, thus may be treated. A therapeutic method involves *in vivo* administration of a blocking antibody to a mammal in an amount effective in inhibiting a TRAIL-deficit biological activity. Disorders caused or exacerbated by TRAIL, directly or indirectly, are thus treated. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

Antibodies raised against a TRAIL receptor, for example, may be screened for agonistic (*i.e.*, ligand-mimicking) properties. Such antibodies, upon binding to cell surface TRAIL receptor, induce biological effects (*e.g.*, transduction of biological signals) similar to the biological effects induced when TRAIL receptor binds to cell surface TRAIL.

Compositions comprising an antibody that is directed against TRAIL receptors or inhibitors, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for compositions containing TRAIL proteins. Also provided herein are conjugates comprising a detectable (*e.g.*, diagnostic) or therapeutic agent, attached to an antibody directed against TRAIL.

In addition, the present invention provides other useful compositions for binding TRAIL receptor or inhibitor nucleic acids, including antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target TRAIL receptor mRNA (sense) or TRAIL receptor DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of, for example, a TRAIL receptor DNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to

about 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein, is known in the art

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block transcription or translation of the target sequence by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of TRAIL receptor proteins, permitting normal levels of TRAIL to be restored. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (*i.e.*, capable of resisting enzymatic degradation), but retain sequence specificity to be able to bind to target nucleotide sequences.

Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, or other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In a preferred procedure, an antisense or sense oligonucleotide is inserted into a suitable retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, those derived from the murine retrovirus M-MuLV.

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule. Suitable ligand binding molecules include, but are not limited to, cell surface

receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell. Alternately, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex.

In further embodiments of the present invention, therapeutically effective amounts of TRAIL receptor or inhibitor binding proteins are administered as described above for TRAIL.

EXAMPLES

The present invention is further described in the following examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. The autoimmune scenarios are relevant for many practical situations, and are intended to be merely exemplary to those skilled in the art. These examples are not to be construed as limiting the scope of the appended claims. Thus, the invention should in no way be construed as being limited to the following example, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Examples 1-4 demonstrate the effect of chronic TRAIL blockade in an animal model of rheumatoid arthritis. Examples 5-9 demonstrate the effect of chronic TRAIL blockade in an animal model of multiple sclerosis as determined by its effect on experimental autoimmune encephalomyelitis (EAE) and encephalitogenic T cells.

Example 1 - Recombinant soluble DR5 blocks TRAIL-induced apoptosis of tumor cells.

In order to determine the biological roles of TRAIL *in vivo* and *in vitro*, large quantities of soluble DR5 (sDR5) were produced using the yeast *Pichia pastoris* system (Higgins, In Current protocols in protein science, Coligan, ed., J. Wiley & Sons, New York, 1998). To generate recombinant soluble DR5, the cDNA that contained the full-length extracellular domain of the human DR5 (Wu *et al.*, *Nature Genetics* 12 (2):141-143 (1997) was cloned into pPIC9K that contains a P_{AOX1} promotor and a six-histidine

tag. (Note that pPIC9K is essentially the same as pGAPZa (Invitrogen, Carlsbad, CA) for the purposes of this invention, and the two plasmids are to be considered to be interchangable.) Several recombinant *Pichia pastoris* clones were generated via homologous recombination (Higgins, 1998), which secreted high levels of sDR5 (up to 25 mg per liter of yeast culture). The sDR5 used in this study was purified through a nickel ion column, then depleted of lipopolysaccharides (LPS) by incubation with polymyxin B agarose (Sigma, St. Louis, MO).

The sDR5 consists of only the extracellular domain of the human TRAIL receptor DR5, and effectively blocks TRAIL-induced apoptosis of tumor cells (Figure 1). The purity of the sDR5 was confirmed by polyacrylamide gel electrophoresis (PAGE). The sDR5, which had a molecular weight of 26 kD, was the only protein band present. The purified sDR5 contained 1-2ng LPS per mg of protein as determined by Limulus amebocyte lysate (LAL) assay. This is comparable to the LPS level in bovine serum albumin (BSA) or human serum albumin (HSA) used in this study, which is 1-4 ng per mg of protein. In previous experiments, it had been determined that neither BSA or HSA had any effect on collagen-induced arthritis using LPS-free PBS as control (Zhang *et al.*, *J. Clinical Investigation* 100:1951-1957 (1997)).

In vitro, purified sDR5 did not induce proliferation of lymphocytes regardless of its concentration in the culture (1-100 μg/ml) (unpublished data).

A recombinant adenovirus carrying the murine TRAIL gene (Ad-TRAIL) was generated by inserting the cloned TRAIL full-length cDNA into the plasmid pAd-tet, followed by homologous recombination with human adenoviral DNA (Zhang *et al.*, 1997). Briefly, the murine full-length TRAIL cDNA was generated from mouse spleen by PCR using specific primers corresponding to the 5' and 3' ends of the coding regions of the TRAIL gene. After adding A-overhangs by incubation with Taq polymerase, the PCR fragment was inserted into the expression vector pCRII-TOPO, which possesses T-overhangs, to create pCRII-TOPO-TRAIL. After amplification of the pCRII-TOPO-TRAIL vector DNA, the TRAIL gene was cut out with Not I, and inserted into the Not I site of the vector pAdtet to create pAdtet-TRAIL.

The ClaI-digested Ad5 genomic DNA was then prepared from H5.000CMVEGFP, a recombinant adenovirus that is depleted of E1a, E1b and a portion

of E3 region, but which contains the green fluorescent protein (GFP) cDNA. The recombinant virus was produced by co-transfecting 293 cells with pAdtet-TRAIL and ClaI-digested Ad5 genomic DNA. White plaques of recombinant viruses were expanded and screened by PCR using TRAIL-specific primers. The recombinant virus-carrying TRAIL gene was then propagated, purified through a cesium chloride gradient, and de-salted on Econo-Pac 10DG column (Bio-Rad, Hercules, CA).

Human Jurkat T cells (clone E6-1) 7500 cells/ml, and K562 B cells, 1 x 10^6/ml, were cultured in RPMI 1640 medium containing various concentrations of TRAIL (Biomol Research Laboratory, Plymouth Meeting, PA), with or without sDR5. The cells were treated as follows: A) 5 μg/ml of BSA; B) 100 ng/ml of TRAIL; C) 5 μg/ml of sDR5 or D) 100 ng/ml of TRAIL + 5 μg/ml of sDR5. Three days later, 2 mg/ml 3-4.5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) was added to the culture, and cell viability was determined by spectrometry at 575 nm wavelength.

The percentage of apoptotic cells was determined as shown in Figures 1A-1D. Apoptosis was determined after eighteen (18) hours by flow cytometry using Annexin-V-FITC per manufacturer's instructions (Pharmingen, San Diego, CA). The percentage of live cells was calculated by assuming the survival rate of untreated cells as 100%.

Although the data is not shown, tests were also conducted in parallel experiments demonstrating that sDR5 had no effect on either Fas- or ultraviolet-induced apoptosis of Jurkat cells. *In vitro*, Ad-TRAIL induced apoptosis of a number of tumor cell lines including Rat-1 cells and 293 cells; addition of sDR5 to the culture prevented Ad-TRAIL-induced apoptosis (Göke and Chen, unpublished).

K562 cells were also treated with various concentrations of TRAIL with (open circle) or without (filled square) 5 μg/ml of sDR5. The results are shown in Figure 1E.

Example 2 - Roles of TRAIL in autoimmune arthritis.

The effect of TRAIL-blockade *in vivo* was first examined in a mouse model of rheumatoid arthritis (Figure 2). Arthritis was monitored by both clinical examination and histochemistry.

Two groups of six- to eight-week old male DBA/1 mice (Jackson Laboratory, Bar Harbor, ME), 4-6 mice per group, were immunized on days 0 and 21 with chicken type II collagen (Sigma, St Louis, MO). The mice were immunized by multiple intradermal

injections of 100 μg chicken type II collagen in 100 μl phosphate buffered saline (PBS) emulsified in an equal volume of complete Freund's adjuvant containing 1mg/ml of *Mycobacterium tuberculosis* H37 RA (Difco, St. Louis, MO). Mice were rechallenged with the same antigen preparation subcutaneously on the flanks 21 days later.

Starting from the day of second immunization (at day 21), mice received daily intraperitoneal injections of 50-300 μg sDR5 or 100 μg bovine serum albumin (BSA) in 0.5 ml PBS for a total of 21 days (Figure 2A). Mice were examined daily for signs of joint inflammation, and scored as follows:

0 = Normal;

1 = Erythema and mild swelling *confined* to the ankle joint or mid-foot;

2 = Erythema and mild swelling *extending* from the ankle to the mid-foot;

3 = Erythema and moderate swelling *extending* from the ankle to the metatarsal joints; or

4 = Erythema and severe swelling *extending* from the ankle to the digits.

The maximal disease score per foot was 4, and the maximal disease score per mouse was 16. The mean disease score per group was calculated as: total disease scores from all animals in the group / the number of animals in the group. (In parallel experiments, other control proteins, such as human or bovine serum albumin, were also used. Similar results to those reported were observed.)

Figure 2A depicts the disease courses in mice treated with of BSA (open square) or sDR5 (filled circle). Each data point represents a mean ± standard deviation (SD) from a total of 5 mice (for sDR5-treated group) or 6 mice (for BSA-treated group). The experiments were repeated five times with similar results.

The differences between the two groups were found to be statistically significant ($P<0.001$) as determined by Mann-Whitney test. As shown in Figure 2A, mice that received the control protein developed typical arthritis, which started approximately 5-10 days after the second immunization, and reached a maximal disease score of 7.8 by day 33. By contrast, in mice treated with sDR5, arthritis was significantly exacerbated. The mean day of onset in the sDR5-treated group was 7.5 ± 1.9 (days post second immunization), as compared to 14.5 ± 1.8 in the control group ($P<0.01$ as determined by

ANOVA). The maximal disease score was increased from 7.8 in the control group to 12.4 in the sDR5-treated group.

Figure 2B depicts the dose-dependent effect of sDR5 on arthritis as judged by disease scores of individual feet taken 12 days after the second immunization. It was apparent that sDR5 enhanced arthritic inflammation in most feet in a dose-dependent manner, indicating that TRAIL is an inhibitor of autoimmune arthritis. A total of 4 groups of mice are shown: one was treated with 100 μg BSA, while the other three were treated with 50-300 μg sDR5. Each data point represents an individual foot, with 16 to 24 feet per group. The differences between BSA- and sDR5-treated groups were found to be statistically significant as determined by ANOVA ($P<0.05$ for mice treated with 50μg sDR5 and $p<0.01$ for mice treated with 100 or 300 μg sDR5).

To directly test TRAIL's ability to inhibit or ameliorate autoimmune arthritis, intra-articular TRAIL gene transfer was performed using a replication-defective adenovirus carrying the mouse TRAIL gene. The virus was injected directly into arthritic joints 6 days after disease onset, as previously described (Zhang *et al.*, 1997). As shown in Figure 3, arthritis was dramatically ameliorated by TRAIL gene transfer following intra-articular injection of 10^{10} recombinant TRAIL viruses, while injection of 10^{9} recombinant TRAIL viruses had only a mild effect.

DBA/1 mice, 4 mice per group, were immunized with type II collagen as described above. Six (6) days after disease onset (10 days after the second immunization), mice were injected intra-articularly and periarticularly to the ankle and tarsal joints of the hind feet as follows: 1) 10 μl PBS (control); 2) 10^{10} particles of adenovirus (Ad) vector; 3) 10^{9} particles of TRAIL virus; or 4) 10^{10} particles of TRAIL virus in 10 μl of PBS as described by Zhang *et al.*, 1987. (The Ad vector contains no TRAIL gene, but is otherwise identical to the TRAIL virus.) Starting from the first day of virus injection, two groups of mice, one non-treated and the other injected with 10^{10} particles of TRAIL virus, were subjected to daily intraperitoneal injections of 100 μg sDR5 for a total of 14 days. The data presented represent disease scores of individual hind feet, 6 days after viral injection. A total of 8 hind feet per group are shown.

Only mice receiving 10^{10} particles of TRAIL virus showed significant improvement ($P<0.001$, as determined by ANOVA). Results are representative of two experiments.

The effect of the treatment was TRAIL-specific, since it could be neutralized by sDR5 (Figure 3). The therapeutic effect of the TRAIL virus lasted about 10 days, and arthritis returned approximately 2 weeks after TRAIL virus injection. As expected, injection of sDR5 after the disease onset also exacerbated the inflammation (Figure 3).

Histochemical analysis of the mice treated in Figure 2 revealed dramatic differences between the groups. DBA/1 mice, 8-9 mice per group, were immunized for arthritis and treated with sDR5 or BSA as shown in Figure 2A. The mice depicted in Figures 4A-4D, 4G and 4H were sacrificed 32 days after the second immunization, and their ankle joints were analyzed for histology and apoptosis.

In the histochemical analysis, the test animals were sacrificed and paws were first fixed in 10% formalin, decalcified in hydrochloric acid, then embedded in paraffin. Joint sections (6 μm) were then prepared and stained with hematoxylin and eosin (HE). The degree of arthritic inflammation was scored as follows:

0 = no signs of inflammation;

1 = mild synovitis;

2 = severe synovitis;

3 = severe synovitis with mild cartilage and bone destruction; or

4 = severe synovitis with severe cartilage and bone destruction.

For detection of apoptotic cells, ApopTag system was used (Oncor, Gaithersburg, MD). Briefly, synovial tissues were snap-frozen and cryosectioned (6 μm). The 3'-OH ends of fragmented DNA were labeled with digoxigenin (DIG)-conjugated nucleotide using terminal deoxynucleotidyl transferase. The randomly incorporated nucleotide polymers were then detected by peroxidase-labeled anti-DIG antibody and chromogen diaminobenzidin. Counterstaining was performed with methyl green. The apoptotic index was recorded as follows:

0 = < 1% of cells in the synovium are apoptotic;

1 = 1-3% of cells in the synovium are apoptotic;

2 = 3.1-5% of cells in the synovium are apoptotic; or

3 = >5% of cells in the synovium are apoptotic.

By comparison, proliferating cells were labeled *in vivo* in the mice depicted in Figures 4E and 4F with bromodeoxyuridine (BrdU). Mice were immunized twice with chicken type II collagen as described above, but starting from the second immunization, mice received daily intraperitoneal injections of 0.8 mg BrdU in 0.5 ml PBS. Mice were sacrificed 21 days after the second immunization, and their synovial joints collected and embedded in paraffin. Synovial sections (6 μm) were then stained with rat anti-BrdU antibody and peroxidase-labeled goat anti-rat IgG as described (Wilson, In *Immunochemical protocols*, vol. 80, Pound (ed.), Humana press, 251-255 (1998).

Control antibodies and tissues were routinely used to exclude non-specific staining.

In the mice treated with the control protein, arthritis was characterized by leukocyte infiltration, mild synovitis and pannus formation (Figures 4A, 4C); cartilage destruction and bone erosion occurred only in a small number of synovial joints. Figure 4A depicts an ankle joint of a BSA-treated mouse with a pathology score of 2 (HE staining, original magnification x20). An arrow indicates signs of synovitis in the joint. Figure 4C depicts an ankle joint of a BSA-treated mouse with a pathology score of 1 (HE staining, original magnification x100). An arrow indicates signs of synovitis.

By contrast, in mice treated with sDR5, severe synovitis, hyperplasia of synovial membrane and cartilage/bone destruction were observed in most synovial joints of the feet (Figures 4B, 4D), which lasted for more than 3 weeks, with little signs of remodeling or fibrosis. Figure 4B depicts an ankle joint of a sDR5-treated mouse with a pathology score of 4 (HE staining, original magnification x20). Arrows indicate severe synovitis in the joint and hyperplasia in the joint, as well as cartilage and bone destruction. Figure 4D depicts an ankle joint of a sDR5-treated mouse with a pathology score of 4 (HE staining, original magnification x100). Arrows indicate severe synovitis and hyperplasia in the joint, as well as cartilage and bone destruction.

To directly label proliferating cells in the joint, the mouse models were treated with nucleotide analogue bromodeoxyuridine (BrdU), then BrdU incorporation was examined by immunohistochemistry. As shown in Figures 4E and 4F, $BrdU^+$ cells were detected in both BSA- and sDR5-treated mice. Figure 4E depicts an ankle joint of a

BSA-treated mouse with a disease score of 2 (BrdU staining, original magnification x400). Arrows indicate $BrdU^+$ nuclei. Figure 4F depicts an ankle joint of a sDR5-treated mouse with a disease score of 3 (BrdU staining, original magnification x400). However, the number of $BrdU^+$ cells in sDR5-treated mice was markedly increased as compared those in BSA-treated mice.

Figure 4G depicts an ankle joint of a BSA-treated mouse with a pathology score of 4 (apoptotic staining, original magnification x200). Arrows indicate apoptotic cells. Figure 4H depicts an ankle joint of a sDR5-treated mouse with a pathology score of 4 (apoptotic staining, original magnification x200).

Mice were treated and sacrificed, corresponding to the treatment in Figure 4. Their paws examined for the degrees of inflammation and apoptosis as described above. A minimum of 3 comparable synovial sections were analyzed per mouse. A total of 8 mice (for sDR5-treated group) and 9 mice (for BSA-treated group) were used. Quantitative analysis of the histochemical data revealed substantial differences between sDR5- and BSA-treated groups (pathology score 3.9 vs. 2.8, Figure 5A).

In determining the pathology scores (Figure 5A), the differences between the two groups were found to be statistically significant, as determined by ANOVA ($p<0.01$). For measuring the apoptotic index (Figure 5B), the differences between the two groups were not statistically significant, as determined by ANOVA ($p = 0.21$).

As shown in Figure 4G and 4H, apoptotic cells were readily detectable in arthritic synovia of both control and sDR5-treated mice 32 days after the second immunization. However, no statistically significant differences between the two groups were observed when the degrees of apoptosis were compared (Figure 5B). In parallel experiments, mice were also sacrificed 14 and 21 days after the second immunization, and examined for apoptosis as in Figures 4G and 4H. Again, no significant differences in the degree of apoptosis were observed between control and sDR5-treated groups (data not shown).

Example 3 - Roles of TRAIL in autoimmune T and B cell responses *in vivo*.

Collagen-induced arthritis is initiated by collagen-specific lymphocytes. To determine whether exacerbation of arthritis by sDR5 is associated with functional alterations of collagen-specific lymphocytes, both cellular and humoral anti-collagen immune responses were examined.

DBA/1 mice, 5-6 mice per group, were treated as in Figure 2A. To test cellular immune responses (Figures 6A-6C), mice were sacrificed 32 days after the second immunization and their inguinal lymph nodes collected. Notably, the short-term TRAIL-blockade in DBA/1 mice did not alter the structure or cellular composition of lymphoid organs as judged by histochemistry and flow cytometry (unpublished data).

For cytokine assays, lymph node cells, 1.5 x 10^6/well, were cultured in 0.2 ml of serum-free medium (X-vivo 20, Biowhittacker, Walkersville, MD), with or without 10-50 μg/ml of chicken type II collagen (CII) or 1 μg/ml of Con-A. Concanavalin (Con)-A-treated cultures were included as positive controls to illustrate the levels of polyclonal T cell responses. Culture supernatants were collected 40 hours later, and IL-2/IFN-γ concentrations were determined by sandwich ELISA, as described by Chen *et al., J. Immunol.* 155:910-916 (1995).

To test lymphocyte proliferation (Figure 6A), lymph node cells, 0.5 x 10^6 cells/well, were first cultured for 72 hours, and then pulsed with ^{3}H-thymidine for an additional 16 hours. Radioactivity [presented as counts per minute (CPM)] was determined using a Wallac beta-plate counter. Figure 6B depicts IL-2 production, while Figure 6C depicts IFN-γ production. Remarkably, both lymphocyte proliferation and cytokine production were enhanced in mice treated with sDR5

In parallel experiments, to test humoral immune responses (Figures 6D-6E), mice were bled retroorbitally on days 14 and 32 after the second immunization, and tested as in the Figures 5A-6C. Anti-collagen antibody responses were determined by ELISA, using chicken type II collagen as antigen. Figure 6D depicts anti-collagen IgG2a titers; Figure 5E depicts anti-collagen IgG1 titers. Each data point represents a mean ± SD from 5 mice (for sDR5-treated group) or 6 mice (for BSA group). The experiments were repeated three times with similar results.

In the collagen-specific analyses, anti-collagen IgG2a was seen to be dramatically increased in mice treated with sDR5, whereas anti-collagen IgG1 was only moderately increased on day 14. Similar differences between control and sDR5-treated mice were observed when a similar ELISA was performed using sDR5 as detecting antigen; no anti-sDR5 antibodies were detected (data not shown). Additionally, sDR5 had no effect on LPS-induced proliferation of splenocytes *in vitro* (unpublished data). These results

indicate that chronic TRAIL-blockade in mice enhanced both cellular and humoral immune responses, which could, in turn, exacerbate autoimmune arthritis.

Example 4 - Roles of TRAIL in apoptosis and cell cycle progression.

TRAIL is unique in that it has been shown to induce apoptosis of some, but not all, tumor cell lines. It is believed that the effect of sDR5 on arthritis can be explained by its blockade of TRAIL-induced apoptosis of inflammatory cells. To test this theory, the effect of TRAIL-blockade on apoptosis of synovial cells was examined *in vivo, i.e.*, the inhibition of DNA synthesis and cell cycle progression by TRAIL was evaluated.

Splenocytes were prepared from 6-8 week old BALB/c mice (Jackson Laboratory), and cultured in DMEM for 3 days in the presence of 10% fetal bovine serum (FBS), activated with 2.5 μg/ml of Con-A. Live cells were then purified through a Ficoll gradient, and cultured in 96-well plates at 3 x 10^5/well in 200 μl of DMEM containing 10% FBS, with or without the following reagents: 1) 100 ng/ml of TRAIL; 2) 5 μg/ml of sDR5; 3) 5 μg/ml of anti-CD95L monoclonal antibody (mAb) (MFL-3); and 4) anti-mouse CD3 mAb (which was coated on the plate by pre-incubating the plate with 10 μg/ml of the antibody at 4°C for 16 hr).

Apoptosis and cell cycle progression were analyzed by flow cytometry using the DNA dye propidium iodide (Noguchi, P.D., In Current Protocols in Immunology. J.E. Coligan, ed., Sarah Greene, New York (1993)). For apoptosis and cell cycle analyses (Figures 7A-7B), cells were cultured for a total of 24 hours, fixed in 70% ethanol, and stained with 50 μg/ml of PI. For thymidine incorporation assays (Figures 7C-7D), cells were cultured for 24 hr, pulsed with 10 μCi/ml of ^{3}H-thymidine for an additional 16 hr. Cells were then harvested and radioactivity determined using a Wallac beta-plate counter.

Figure 7A shows the percentage of apoptotic cells as determined by flow cytometry. As shown, anti-CD3 mAb induced apoptosis of approximately 16% of the cells. This was completely prevented by anti-CD95L mAb, confirming an essential role for CD95L in activation-induced cell death (AICD) (Ju *et al., Nature* 373:444-448 (1995); Dhein *et al., Nature* 373:438-441 (1995); Brunner *et al., Nature* 373:441-444 (1995)). By contrast, sDR5 moderately increased AICD induced by anti-CD3 mAb (Figure 7A).

The spontaneous apoptotic rate in cultures containing no anti-CD3 mAb was 12%, which was subtracted from the present data. For cultures that contained TRAIL and anti-CD3 mAb, the percentage of anti-CD3-induced apoptosis was 16 ±1%, which was comparable to that of the cultures treated with anti-CD3 mAb alone. The differences between anti-CD95L mAb treated culture and all other cultures are statistically significant as determined by ANOVA ($p<0.0001$). Unexpectedly, the total number of cells, especially cells in the S-G2/M phases of the cell cycle, were dramatically increased in cultures containing sDR5. This increase in S-G2/M cells was TRAIL-specific since it was partially blocked by recombinant TRAIL.

Figure 7B depicts the number of S-G2/M cells/well, as determined by flow cytometry. For cultures that contained TRAIL and anti-CD3 mAb, the number of cells in the S-G2/M phases was 70-90% of that of the anti-CD3 mAb treated culture. The total numbers of live cells/well recovered from each group were as follows: cultures with anti-CD3 mAb alone, 0.3×10^5; cultures with anti-CD3 mAb + sDR5, 1.6×10^5; cultures with anti-CD3 mAb + anti-CD95L mAb, 1.1×10^5; cultures with anti-CD3 mAb + sDR5 + TRAIL, 0.6×10^5. The differences between all four groups are statistically significant as determined by ANOVA ($p<0.01$).

These results strongly suggest that although TRAIL may not induce apoptosis of lymphocytes, it does play important roles in regulating cell cycle progression. To directly test this theory, the effect of recombinant TRAIL and sDR5 was examined on cell cycle progression of lymphocytes. T lymphocytes were stimulated with anti-CD3 mAb as in Figure 7A, and G1-to-S phase progression (DNA synthesis) was determined by ^{3}H-thymidine incorporation (cells entering the S phase of the cell cycle synthesize DNA and take up thymidine) (Figures 7C and 7D).

The data presented in Figures 7C and 7D represent the means ± SDs of CPM of triplicate cultures. For cultures containing anti-CD3 mAb, the differences between the two groups are statistically significant as determined by ANOVA ($p<0.01$). The experiments were repeated twice with similar results. The concentrations of TRAIL and sDR5 used in these experiments were selected based on previously performed dose-dependency studies. When 0.2-1 μg/ml of sDR5 was used, less significant effects on cell

cycle progression were observed. Similarly, when 50-150 ng/ml of TRAIL was tested, much less significant effects on cell cycle were detected (data not shown).

As shown in Figures 7C and 7D, anti-CD3 mAb induced a marked increase in thymidine uptake. This was significantly inhibited by TRAIL (Figure 7C), but enhanced by sDR5 (Figure 7D). Again, neither TRAIL nor sDR5 affected apoptosis of cells in the culture (unpublished data). Since AICD is regulated by cell cycle progression, inhibition of cell cycle by TRAIL may modulate the sensitivity of cells to AICD as shown in Figure 7A.

Example 5 - Generation of a recombinant soluble TRAIL receptor that blocks TRAIL function.

The recombinant TRAIL receptor used in this study was the soluble DR5 (sDR5) produced in the yeast *Pichia pastoris* system, as described in Example 1. The cDNA containing the full-length extra-cellular domain of the human DR5 (Wu *et al.*, 1997) was cloned into pGAPZa (Invitrogen, Carlsbad, CA) that contains a P_{AOX1} promoter and a six histidine tag as well as a zeocin resistance gene. Several recombinant *Pichia pastoris* clones with zeocin resistance were generated, which secreted up to 25 mg sDR5 per liter of yeast culture.

The recombinant protein was purified by Ni ion column chromatography and treated with polymyxin B agarose, as in Example 1. The purity of the sDR5 was confirmed by polyacrylamide gel electrophoresis and Coomasie Blue staining. As above, sDR5, at 26 kD, was the only protein band present. The purified sDR5 contains 1-2 ng of LPS per mg of protein as determined by LAL assay. In previous experiments, it had been determined that this level of LPS had no effect on the development of EAE or myelin oligodendrocyte glycoprotein (MOG)-specific immune responses using LPS-free PBS as a control.

To assess the biological activities of recombinant sDR5, the TRAIL-induced apoptosis of mouse L929 cells was studied. As shown in Figure 8, TRAIL induced apoptosis of L929 cells in a dose-dependent manner.

By the method of Song *et al.*, 1997, L929 cells were first cultured in flat-bottom 96-well plate, at 2 x 10^4 cells/well, in 100 µl AIM-V medium (Gibco BRL). Sixteen hours later, actinomycin D was added to the culture at 1 µg/well, and cells were cultured

for another 2 hours. Recombinant TRAIL (Biomol Research Laboratory, Plymouth Meeting, PA) was then added with or without 5 μg/ml of sDR5, and culture was continued for an additional 5 hours. 3-4.5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) was added only for the last hour of the culture to determine the percentage of dead cells.

At the end of the culture, medium was removed and DMSO (100 μl/well) was added, and absorbance was determined at 595 nm. The percentage of dead cells was calculated using untreated cells as control. The survival rate of untreated cells was assumed to be 100%.

As seen in Figure 8, TRAIL induced apoptosis in the L929 cells was completely blocked by addition of recombinant sDR5. Thus, as demonstrated in the parallel experiments in Example 1 in other tumor cell lines, including human Jurkat cells and K562 cells, the recombinant sDR5 is biologically active and can be used to block TRAIL function *in vitro*.

Example 6 -TRAIL-blockade exacerbates MOG-induced autoimmune encephalomyelitis.

To investigate the roles of TRAIL *in vivo*, the consequences of TRAIL-blockade in an animal model of multiple sclerosis were examined. In this example, female C57BL/6 mice, 4-6 week old, were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in the University of Pennsylvania animal care facilities and were acclimated for 5-7 days before being used for experiments.

Mouse MOG38-50 (MOG, peptides 38-50) peptide was synthesized using Fmoc solid phase methods and purified through HPLC by Research Genetics (Huntsville, AL). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). The following reagents were purchased from PharMingen (San Diego, CA): rat anti-mouse IL-2 (clone JES6-1A12), IL-4 (BVD4-1D11), and IFN-γ (R4-6A2) mAb; biotin-labeled rat anti-mouse IL-2 (clone JES6-5H6), IL-4 (BVD6-24G2), and IFN-γ (XMG112D) mAb; recombinant mouse IL-2, IL-4, IL-10 and IFN-γ. Quantitative enzyme-linked immunosorbent assay (ELISA) for IL-2, IL-4, and IFN-γ was performed as per manufacturer's recommendations.

Groups of C57BL/6 mice (6-8 mice per group) were immunized by subcutaneous injection with 400 μg of MOG 38-50 peptide to induce experimental autoimmune

encephalomyelitis (EAE). Prior to administration, the MOG 38-50 peptide was emulsified in complete Freund's adjuvant (CFA) containing 100 μg of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI). Pertussis toxin, 250 ng per mouse, was injected intravenously on the day of immunization, and again 48 hours later. Mice were evaluated daily and scored for (EAE) as follows (Chen *et al., Science* 265:1237(1994).

0 = no disease;

1 = tail paralysis;

2 = hind limb weakness;

3 = hind limb paralysis;

4 = hind limb paralysis plus forelimb paralysis; or

5 = moribund or dead.

The incidence of the disease was more than 90% for all the experiments. Only mice that developed EAE were included for calculating mean clinical scores.

Eight days after disease onset (when approximately 90% of the mice had developed signs of EAE), mice were injected intraperitoneally with either 200 μg of sDR5 or a control protein (HAS), 200 μg/mouse, was injected intraperitoneally once every other day for a total of 17 days. The presented data are representative of three experiments.

As shown in Figure 9A, C57BL/6 mice developed typical EAE, starting approximately 18 days after immunization. Injection of sDR5, performed 7 days after disease onset until the end of the experiment, shown as days 25 to 42, significantly exacerbated the disease. The mean maximal disease score in the control group was 2.0±0.5. This was increased to 3.3±0.4 in the sDR5 treated group. One out of six mice died from EAE in the sDR5 treated group, whereas none died in the control group.

To determine whether the effect of TRAIL-blockade is limited to the effector phase of EAE, the consequences of TRAIL-blockade were also investigated during the inductive phase (days 0-16) of the disease. Thus, MOG-immunized mice were treated with sDR5 or a control protein from the day of immunization until the day of disease onset. One mouse out of 12 developed signs of EAE.

As shown in Figure 9B, no significant differences between control and sDR5 treated groups were observed with respect to disease onset or severity. Thus, the

differences between the two groups are statistically significant as determined by Mann-Whitney test ($P<0.01$) for panel A, but not for B. This suggests that TRAIL-blockade during the inductive phase of EAE alone may not be sufficient to affect the disease course.

Example 7 - TRAIL-blockade enhances the formation of inflammatory lesions in the CNS.

To investigate the effect of TRAIL-blockade on the formation of inflammatory lesions in the central nervous system (CNS), quantitative histopathological studies were conducted to determine inflammation and apoptosis of spinal cords.

Mice were treated as in Example 6, and sacrificed 42 days after immunization. The mice were then perfused with PBS and 10% formalin phosphate. Spinal cords were first embedded in paraffin, cut into 5 pieces, and then sectioned at 5 μm and stained with luxol fast blue and cresyl violet (Moore *et al., Laboratory Invest.* 51:416 (1984).

The total area of tissue section and the area of inflammation were measured using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) in a blinded manner. The percentage of the spinal cord with inflammation was calculated as follows: (area of the spinal cord that is infiltrated by inflammatory cells / total area of the spinal cord sections measured) x 100. A total of ten (10) tissue sections from cervical, thoracic, lumbar and sacral spinal cord were analyzed for each animal.

As shown in Figures 10A (sDR5 treated mice) and 10B (control; BSA treated mice), inflammatory lesions were readily detectable.

Apoptosis was determined by TUNEL staining of spinal cords from sDR5 (Figure 10C) and BSA (Figure 10D) treated mice (original magnification x 200). TUNEL staining was performed on paraffin-embedded formalin-fixed spinal cord sections, as described by Chen *et al., J. Neuroimmunol.* 82:149 (1998). Briefly, sections were de-waxed in xylene, hydrated in water/ethanol and washed in PBS. Endogenous peroxidase activity was inactivated by incubating the tissue in 3% H_2O_2. Fragmented DNA in apoptotic cells was labeled with digoxigenin-conjugated dUTP (Roche Molecular Biochemicals, Indianopolis, IN) using TdT enzyme (Clontech, Palo Alto, CA). The labeled DNA was then detected by peroxidase-conjugated anti-digoxigenin antibody (Roche) using diaminobenzidine as substrate. Counterstaining was performed with

methyl green. The number of apoptotic cells in the lesions was determined by light microscopy. Arrows in Figures 10C and 10D indicate apoptotic nuclei.

The inflammatory lesions consisted mostly of lymphocytes and macrophages as well granulocytes and microglial cells. To quantify the degree of inflammation, the area of spinal cord sections that showed signs of infiltration, as well as the total area of each spinal cord, sections were measured using the Image-Pro Plus software. The degree of inflammation was then evaluated based on the percentage of the spinal cord areas that show signs of inflammation. As shown in Figure 11, the inflammation in the mice treated with sDR5 was significantly more severe, than that seen in the control mice. Each data point represents a percentage of spinal cord section that was inflamed. The horizontal bars represent the means of respective groups. The differences between the two groups are statistically significant as determined by Student *t* test ($p<0.05$). Filled squares represent mice treated with HSA. Open circles represent mice treated with sDR5.

The extent of demyelination correlated well with the degree of inflammation and clinical score. Mice with similar disease scores exhibited similar degrees of demyelination and inflammation, regardless of treatment groups (data not shown).

Example 8 - TRAIL-blockade does not affect apoptosis of inflammatory cells in the CNS.

To test whether the effect of sDR5 on EAE can be explained by its blockade of "TRAIL-induced apoptosis" of inflammatory cells, the effect of TRAIL-blockade on apoptosis of inflammatory cells in the CNS was tested. As shown in Figures 10C and 10D, apoptotic cells were readily detectable in spinal cords of both control and sDR5 treated mice. The vast majority of apoptotic cells were localized within the inflammatory lesions, with a few detected outside the inflamed area. In sections that did not contain inflammatory lesions, no apoptotic cells were detected.

To quantitatively compare the degree of apoptosis between control and sDR5 treated groups in the CNS, the number of apoptotic cells per mm^2 were calculated in spinal cord that showed signs of inflammation. Groups of C57BL/6 mice, 4 mice per group, were treated as in Example 6, and sacrificed 42 days after immunization. Spinal cord was treated and examined for apoptosis as previously described. The numbers of apoptotic nuclei per mm^2 of inflamed tissue were counted and plotted against the

percentages of the corresponding spinal cord sections that were inflamed (degree of inflammation) in the same section.

As shown in Figure 12, no statistically significant differences in the degree of apoptosis were observed between control and sDR5 treated groups as determined by Mann-Whitney test ($p>0.05$). Filled squares represented mice treated with HSA. Open circles represented mice treated with sDR5. These results suggest that TRAIL may not regulate apoptosis of inflammatory cells in the CNS.

Example 9 - TRAIL-blockade enhances anti-MOG T cell responses.

EAE is a T cell-mediated autoimmune disease. To determine whether TRAIL-blockade affected the functions of encephalitogenic T cells, anti-MOG T cell responses were studied in mice following TRAIL-blockade.

Groups of C57BL/6 mice, 6 mice per group, were immunized with MOG peptide to induce EAE. Recombinant sDR5 or HSA, 200 μg/mouse, was injected intraperitoneally once every other day for a total of 16 days as in Example 6. Mice were sacrificed 10 days after the last injection of sDR5, and splenocytes were tested for anti-MOG proliferative and cytokine responses.

For cytokine assays, splenocytes were cultured at 1.5×10^6 cells/well in 0.2 ml of DMEM (Gibco BRL Life Technologies, Grand Island, NY) containing 10% fetal bovine serum and various amounts of MOG38-50 peptide. Culture supernatants were collected 40 hours later and cytokine concentrations determined by ELISA. For proliferation assays, 0.5×10^6 cells/well were used. ^{3}H-thymidine was added to the culture at 48 hr, and cells were harvested 16 hours later. Radioactivity was determined using a flatbed beta counter (Wallac, Gaithersburg, MD).

The data presented are representative of two experiments. Mice were immunized with MOG peptide to induce EAE and treated with either sDR5 or BSA for a total of 16 days. Anti-MOG T cell responses were determined *ex vivo* 10 days after the last injection of sDR5. As shown in Figure 13, splenocytes of BSA-treated mice produced primarily TH1 type cytokines (*i.e.*, IL-2 and IFN-γ) in response to MOG peptide. This was significantly increased in mice treated with sDR5. A small but significant amount of IL-4 was also detected in sDR5-treated group. Interestingly, lymphocyte proliferative

responses were comparable between BSA- and sDR5-treated groups. These results suggest that TRAIL-blockade enhances functions of both TH1 and TH2 type cells *in vivo*.

In summary, the present findings establish that 1) unlike its apoptotic effect on tumor cells, TRAIL inhibits cell cycle progression of non-transformed lymphocytes, and 2) unlike TNF that promotes inflammation, TRAIL inhibits autoimmune inflammation and prevents self-tissue destruction.

Each and every patent, patent application and publication that is cited in the foregoing specification is herein incorporated by reference in its entirety.

While the foregoing specification has been described with regard to certain preferred embodiments, and many details have been set forth for the purpose of illustration, it will be apparent to those skilled in the art that the invention may be subject to various modifications and additional embodiments, and that certain of the details described herein can be varied considerably without departing from the spirit and scope of the invention. Such modifications, equivalent variations and additional embodiments are also intended to fall within the scope of the appended claims.

What is claimed is:

1. A method for achieving or enhancing normal levels of cellular apoptosis in non-tranformed cells of a patient by administering to the patient a therapeutically effective amount of purified TRAIL ligand or active fragment thereof.

2. The method of claim 1, wherein the patient suffers from an autoimmune disease or condition.

3. A method for achieving or enhancing normal levels of cellular apoptosis in non-tranformed cells of a patient by administering to the patient a therapeutically effective amount of an isolated nucleic acid sequence encoding TRAIL or active fragment thereof.

4. The method of claim 3, wherein the nucleic acid sequence is produced recombinantly.

5. The method of claim 2, wherein the method prevents, inhibits or decreases inflammation, tissue damage or injury related to the autoimmune disease or condition.

6. The method of claim 2, wherein the autoimmune disease or condition is selected from the group consisting of arthritis, autoimmune encephalomyelitis, insulin-dependent diabetes mellitus, hemolytic anemias, rheumatic fever, thyroiditis, Crohn's disease, myasthenia gravis, glomerulonephritis, autoimmune hepatitis, multiple sclerosis, systemic lupus erythematosus, and ankylosing spondylitis.

7. The method of claim 2, wherein the autoimmune disease or condition is a result of allogeneic tissue or organ transplant or graft-versus-host disease.

8. The method of claim 6, wherein the autoimmune disease is arthritis.

9. The method of claim 8, wherein the arthritis is induced, rheumatoid or chronic.

10. The method of claim 6, wherein the autoimmune disease autoimmune encephalomyelitis or multiple sclerosis.

11. The method of blocking the activity of an endogenous TRAIL receptor or inhibitor in a patient by administering to the patient a therapeutically effective amount of a purified TRAIL agonist, in an amount sufficient to enhance the patient's level of TRAIL ligand.

12. The method of claim 11, wherein cellular apoptosis in non-tranformed cells of the patient is enhanced or ameliorated by administering to the patient a therapeutically effective amount of an isolated nucleic acid sequence encoding said TRAIL agonist, in an amount sufficient to enhance the patient's level of TRAIL ligand.

13. The method of claim 12, wherein the nucleic acid sequence is produced recombinantly.

14. The method of claim 11, wherein the patient suffers from an autoimmune disease or condition.

15. The method of claim 14, wherein the method prevents, inhibits or decreases inflammation, tissue damage or injury related to the autoimmune disease or condition.

16. The method of claim 14, wherein the autoimmune disease or condition is selected from the group consisting of arthritis, autoimmune encephalomyelitis, insulin-dependent diabetes mellitus, hemolytic anemias, rheumatic fever, thyroiditis, Crohn's disease, myasthenia gravis, glomerulonephritis, autoimmune hepatitis, multiple sclerosis, systemic lupus erythematosus, and ankylosing spondylitis.

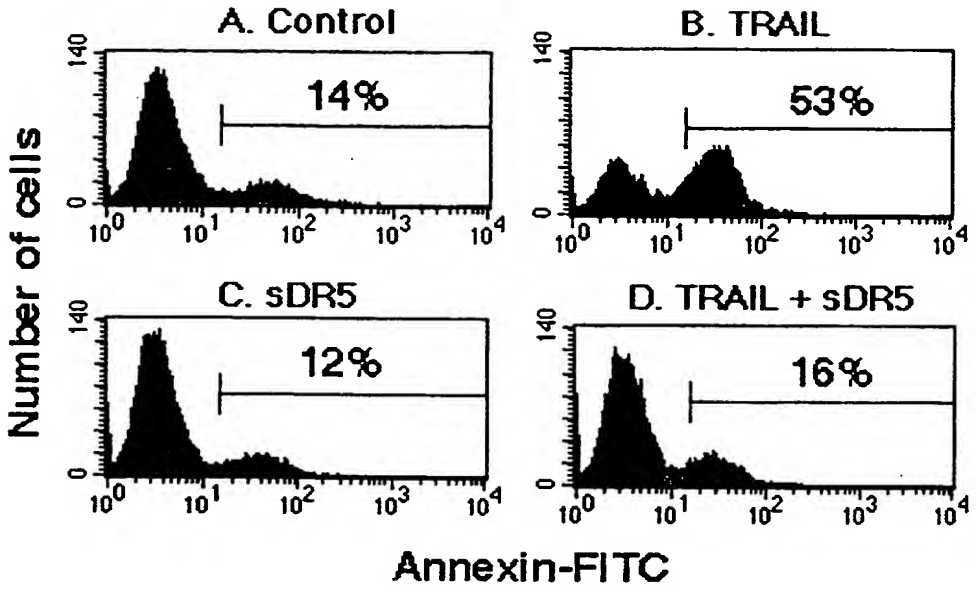
17. The method of claim 16, wherein the autoimmune disease is arthritis.

18. The method of claim 16, wherein the autoimmune disease autoimmune encephalomyelitis or multiple sclerosis.

19. The method of claim 14, wherein the autoimmune disease or condition is a result of allogeneic tissue or organ transplant or graft-versus-host disease.

20. The method of claim 11, wherein the non-transformed cells are selected from the group consisting of lymphocytes, including autoreactive lymphocytes, cytokines and synovial cells.

21. The method of claim 11, wherein the TRAIL agonist is a receptor or inhibitor of a TRAIL receptor or inhibitor, which is selected from the group consisting of antibodies to TRAIL receptors or inhibitors, antisense molecules complimentary to TRAIL receptors or inhibitors, and any molecule which binds to or blocks TRAIL receptors or inhibitors.


A. Control
14%
B. TRAIL
53%
C. sDR5
12%
D. TRAIL + sDR5
16%
Number of cells
Annexin-FITC

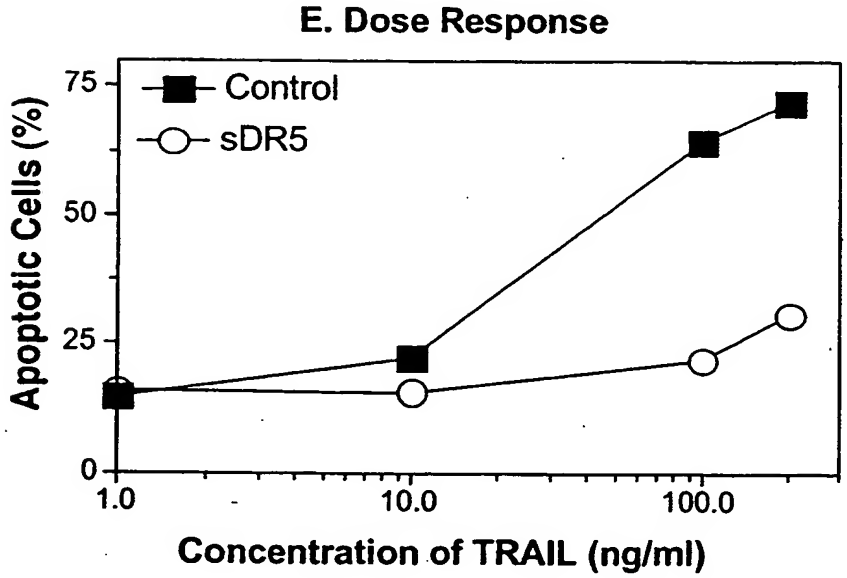

E. Dose Response
Control
sDR5
Apoptotic Cells (%)
Concentration of TRAIL (ng/ml)

FIGURE 1

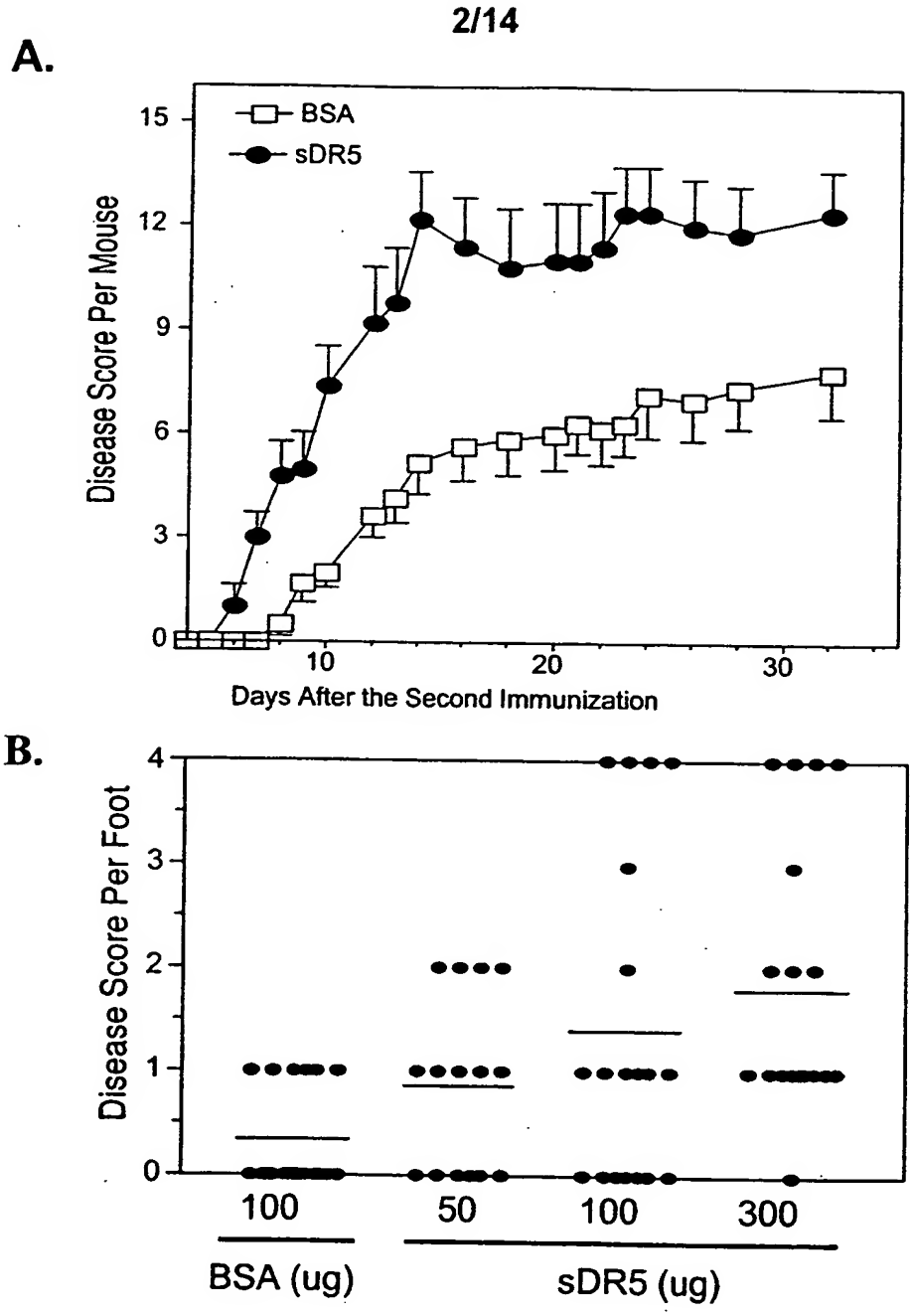

A.
BSA
sDR5
Disease Score Per Mouse
15
12
9
6
3
0
10
20
30
Days After the Second Immunization
B.
Disease Score Per Foot
4
3
2
1
0
100
50
100
300
BSA (ug)
sDR5 (ug)

FIGURE 2

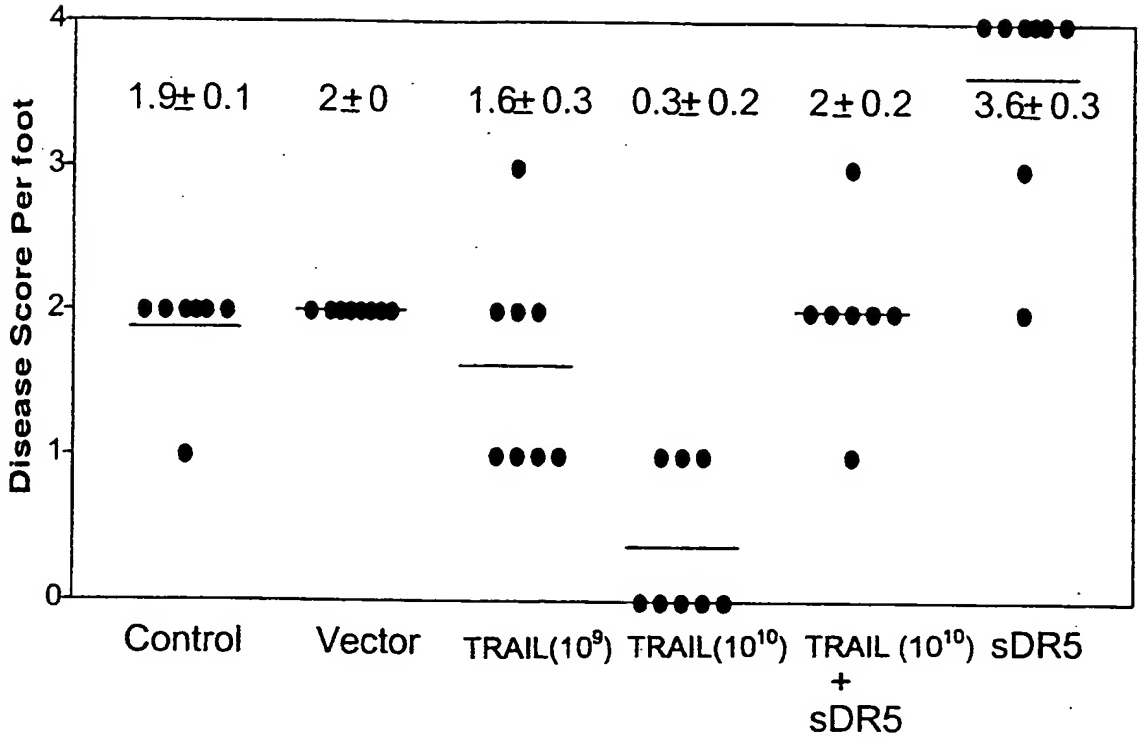


FIGURE 3

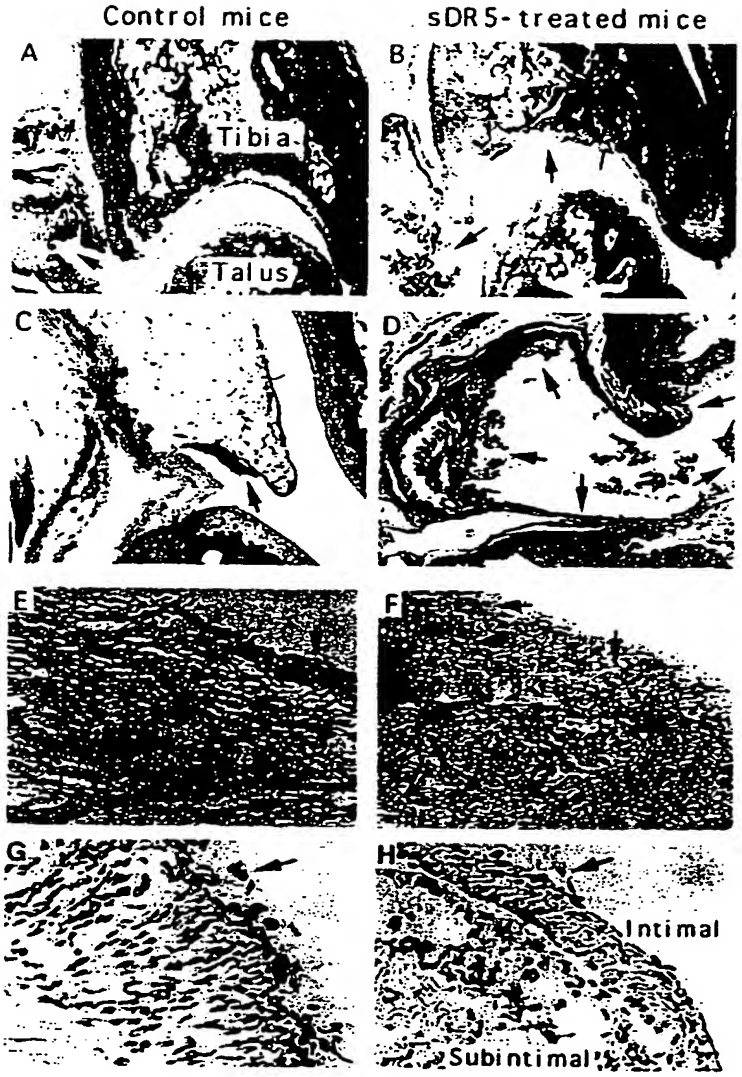


FIGURE 4

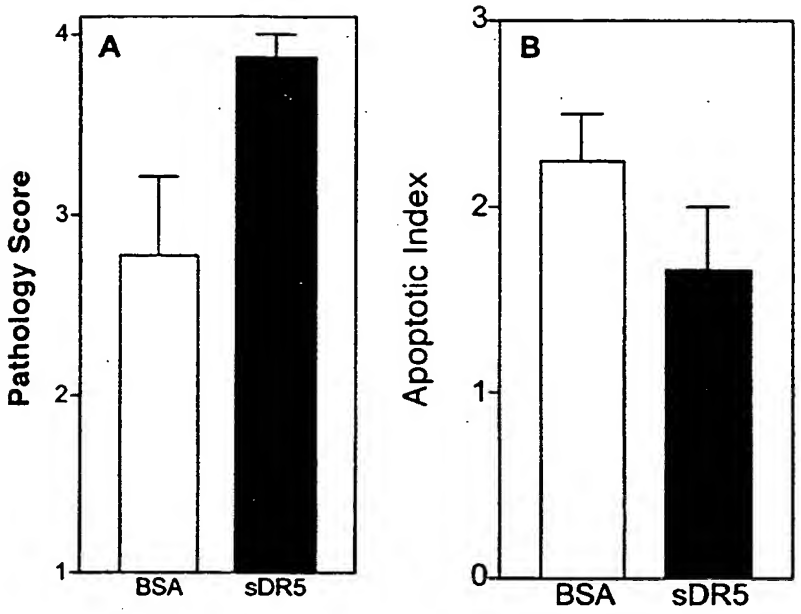
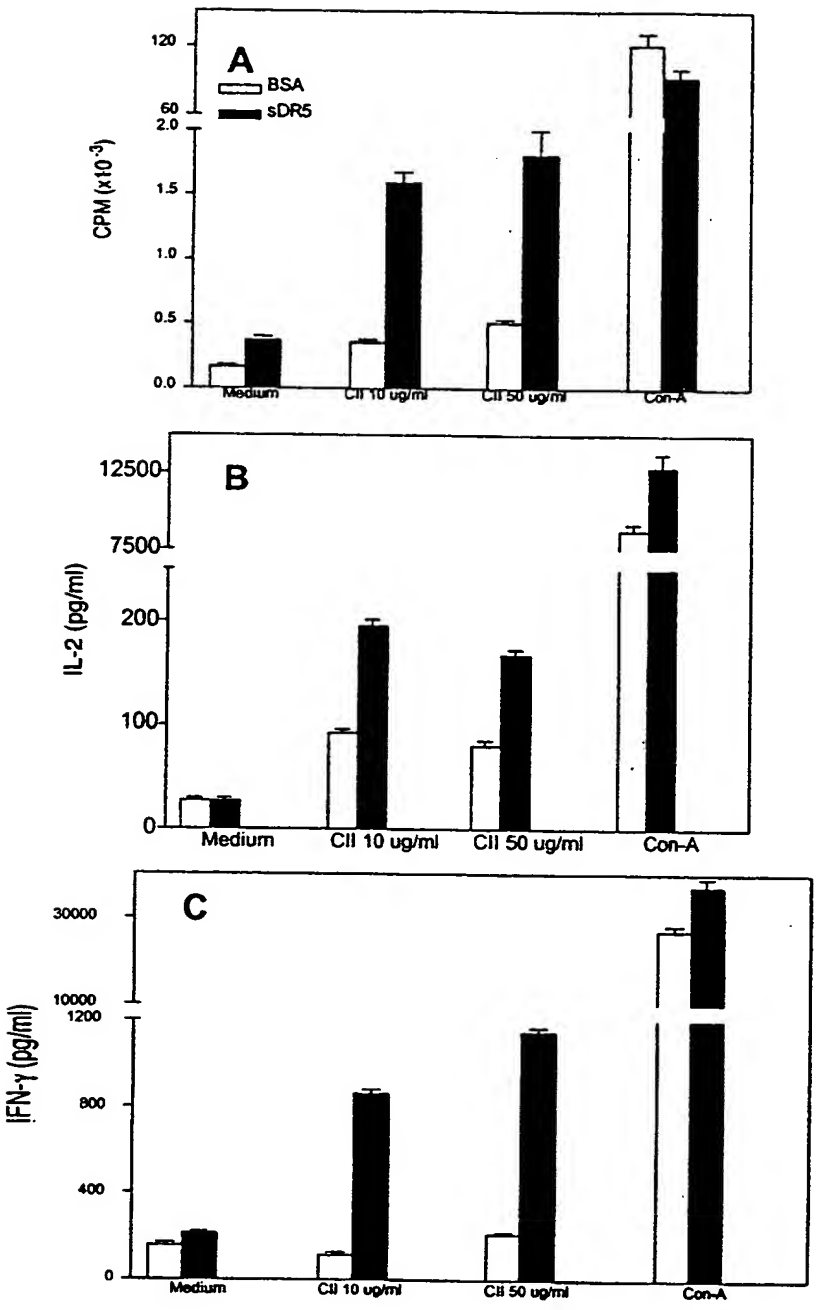
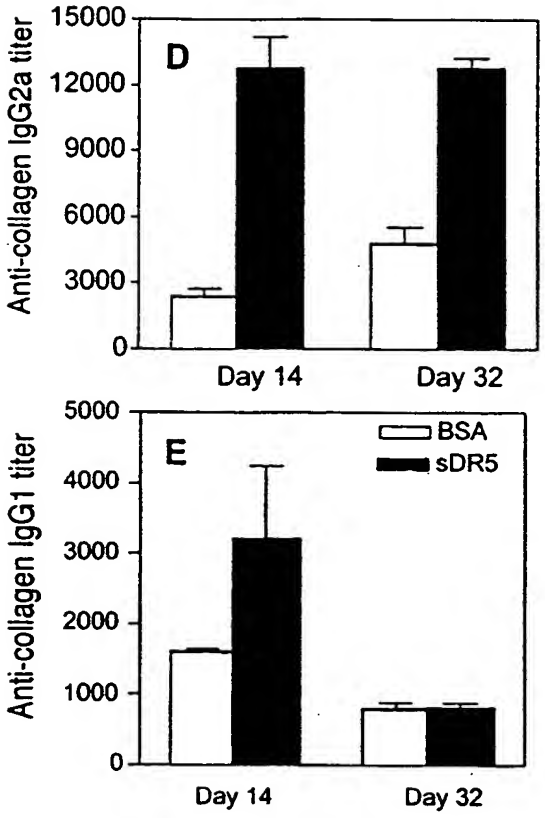


FIGURE 5



FIGURES 6A-6C



FIGURES 6D-6E

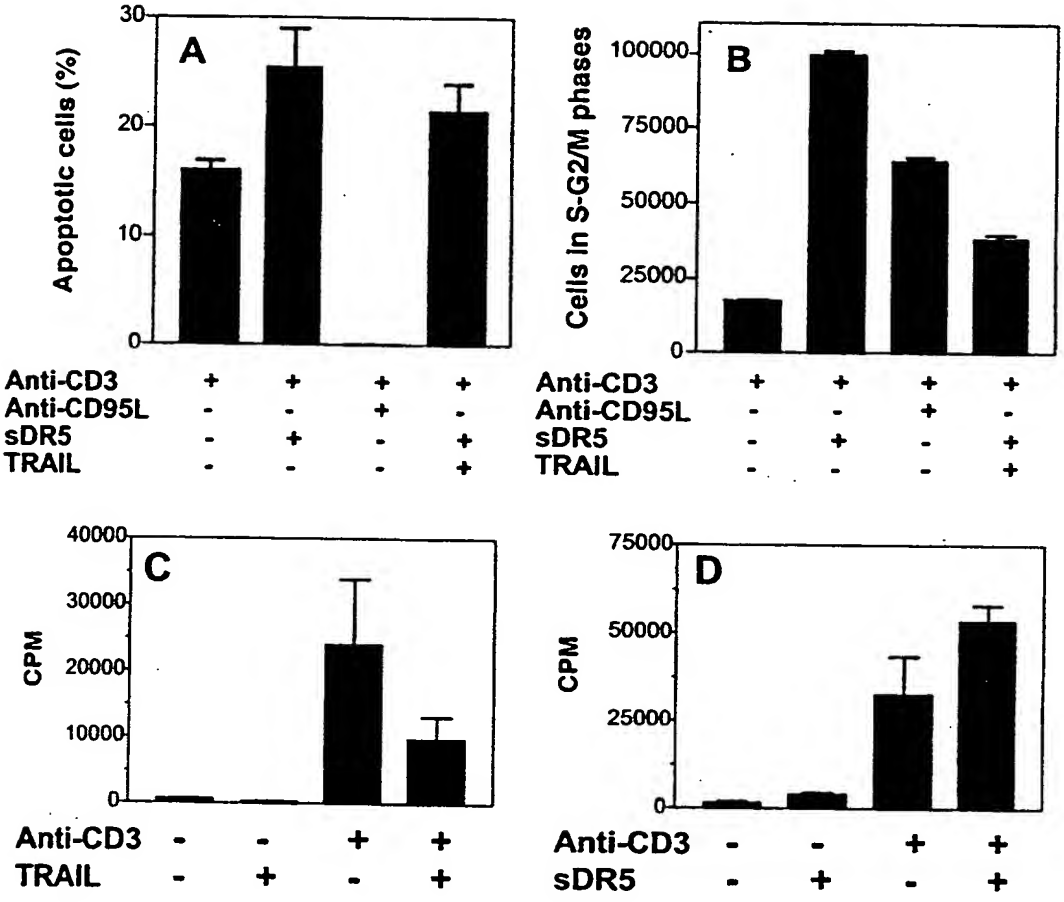


FIGURE 7

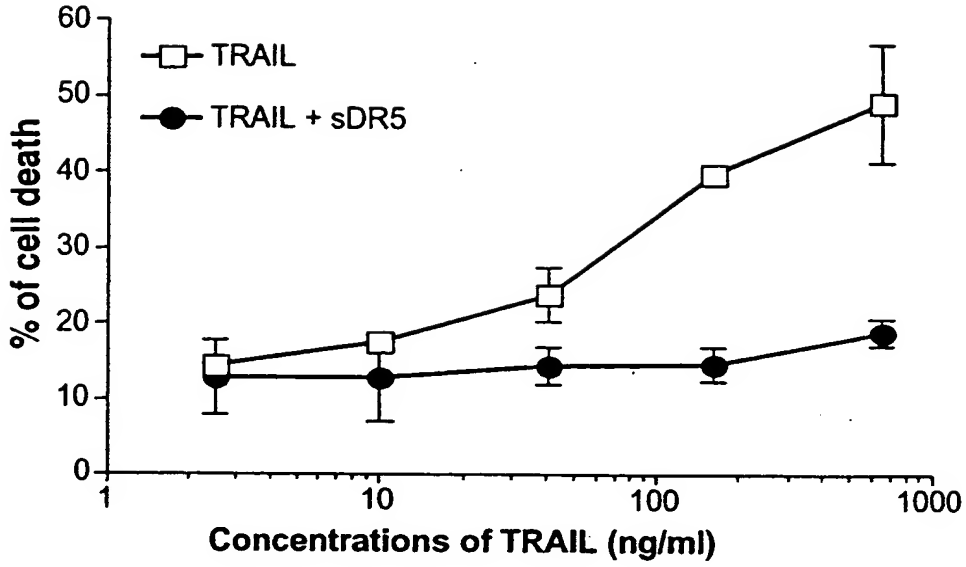
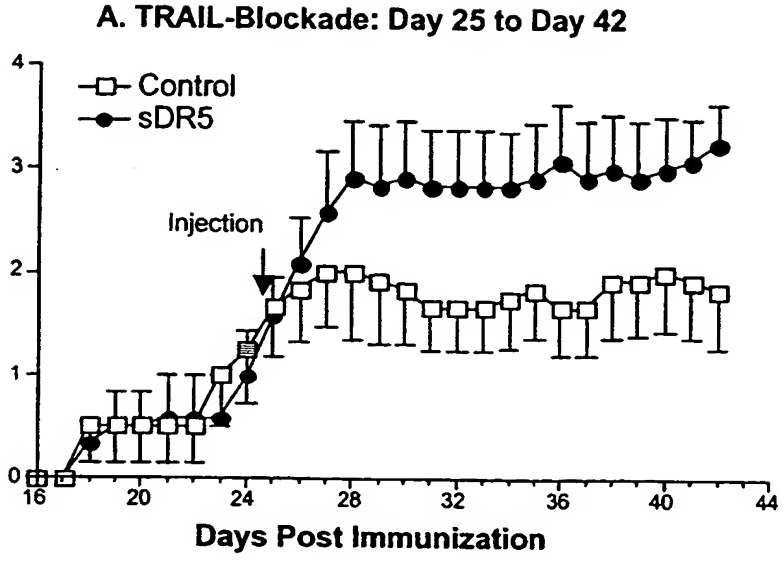


FIGURE 8


A. TRAIL-Blockade: Day 25 to Day 42
Control
sDR5
Injection
0
1
2
3
4
16
20
24
28
32
36
40
44
Days Post Immunization

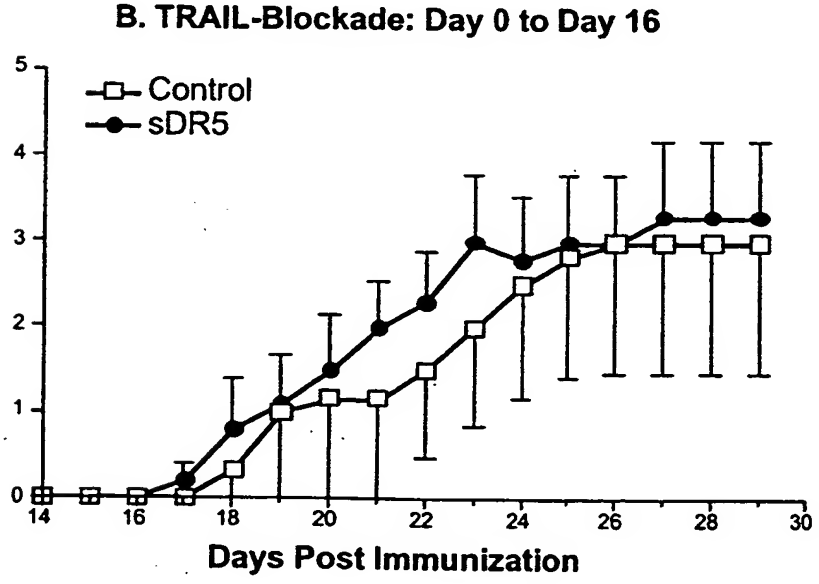

B. TRAIL-Blockade: Day 0 to Day 16
Control
sDR5
0
1
2
3
4
5
14
16
18
20
22
24
26
28
30
Days Post Immunization

FIGURE 9

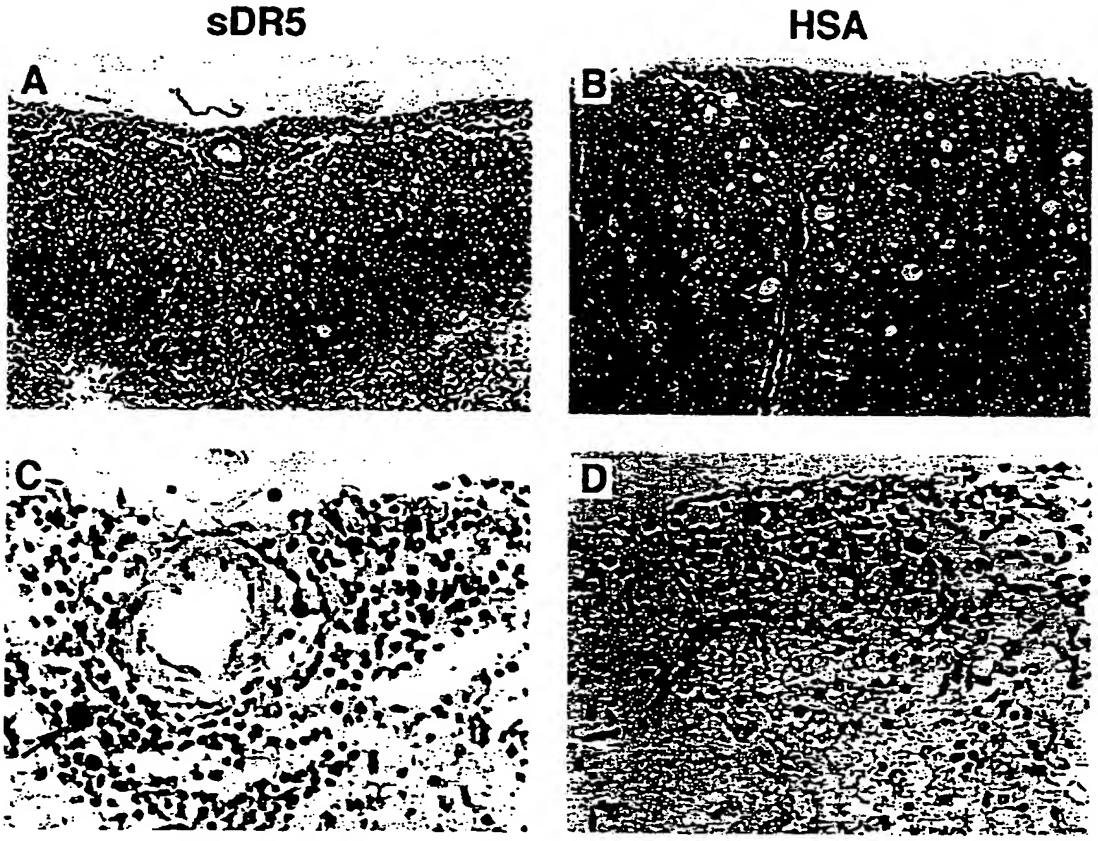


FIGURE 10

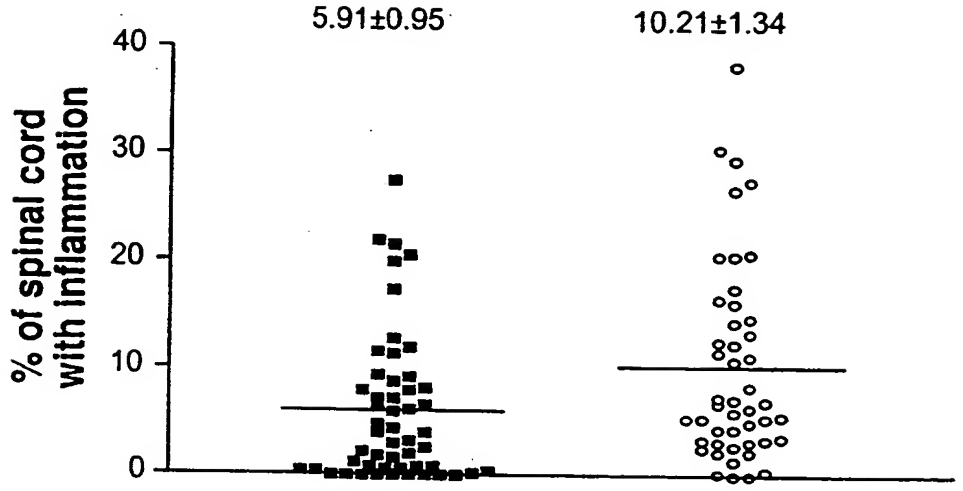

5.91±0.95
10.21±1.34
% of spinal cord with inflammation
40
30
20
10
0

FIGURE 11

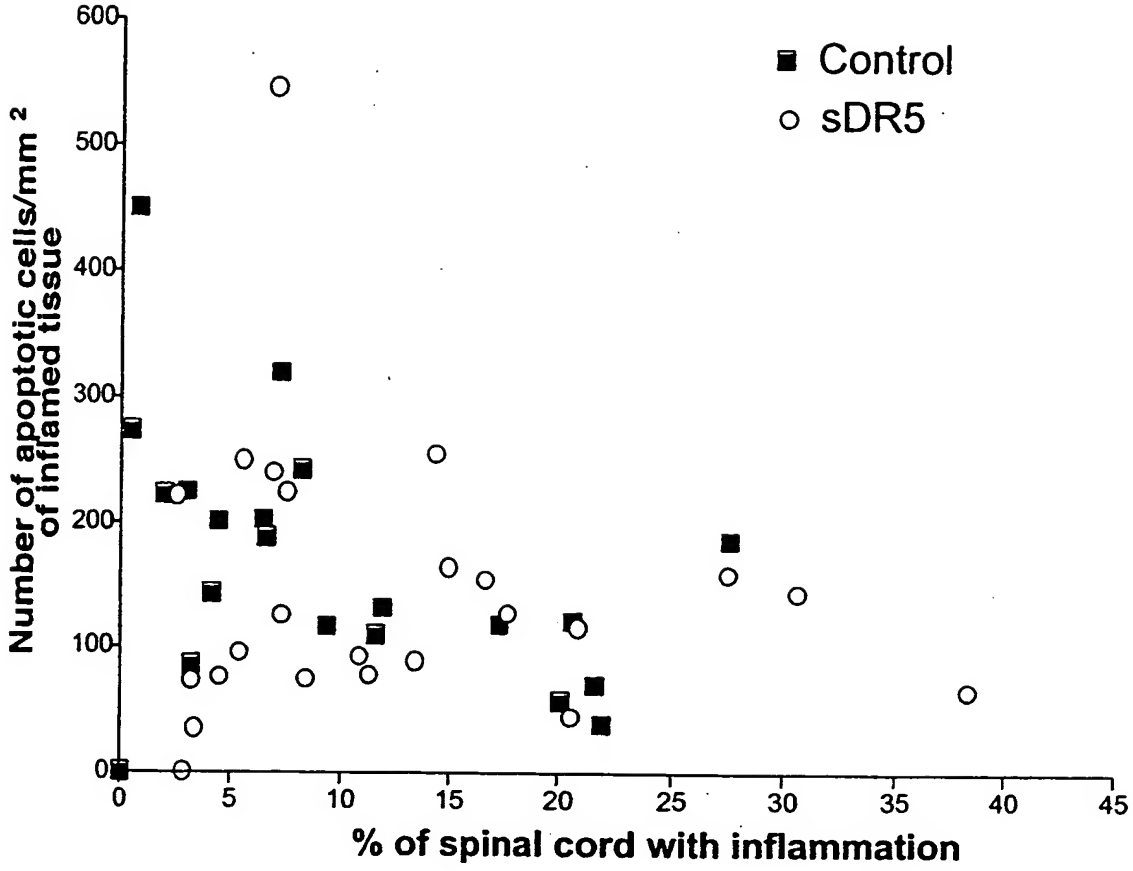

Control
sDR5
Number of apoptotic cells/mm 2 of inflamed tissue
% of spinal cord with inflammation
0
100
200
300
400
500
600
0
5
10
15
20
25
30
35
40
45

FIGURE 12

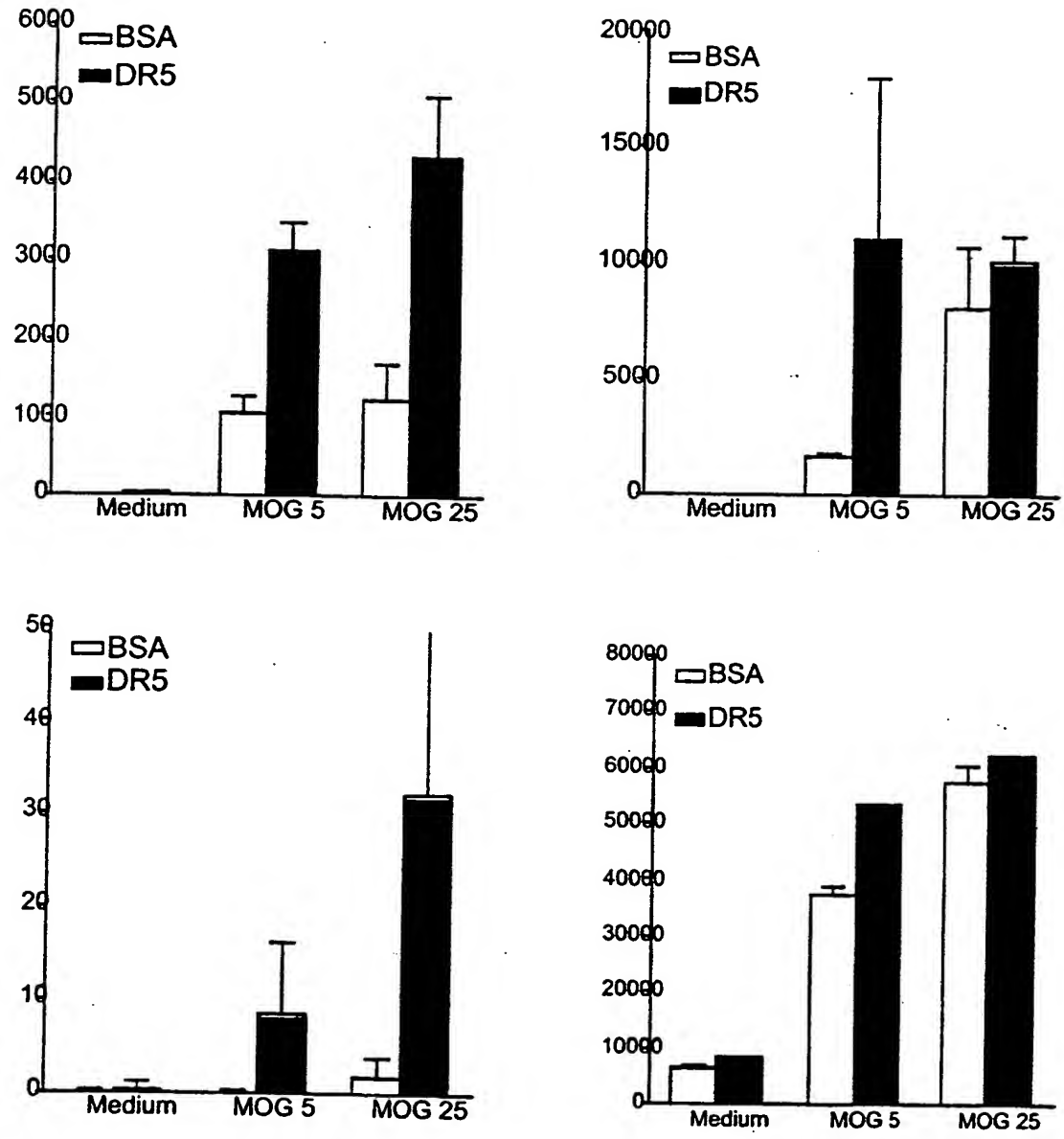


FIGURE 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/26862

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 38/16, 38/17, 48/00
US CL :514/2, 8, 12, 44, 825, 885, 903
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 8, 12, 44, 825, 885, 903

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CAS ONLINE, MEDLINE, CAPLUS
search terms: TRAIL ligand, administer, therapy, method, treatment, agonist, nucleic acid.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,763,223 A (WILEY ET AL) 09 June 1998 (09/04/98), see entire document.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 DECEMBER 2000	Date of mailing of the international search report 31 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PREMA MERTZ Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*